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14. ABSTRACT  This is an Exploration – Hypothesis Development Award (EHD) in which we modeled in a mouse model system the natural androgen decline in the aging male (ADAM) as it occurs after the age of 40 years in humans. ADAM coincides with a dramatic increase in the prostate cancer incidence in all population groups. To date we have collected all biological specimens and are in the process of detailed analyses. We have also explored androgen receptor (AR) signaling in pilot studies to allow a better understanding of this axis in ADAM. Five publications are in press.					
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## **Introduction:**

The androgen receptor (AR), a ligand-dependent nuclear transcription factor, plays a vital role in prostate cancer (PCa) development by regulating the transcription of target genes involved in cell cycle regulation and apoptosis. Physician-induced androgen ablation (castration or AR inhibition) selects for aberrant AR signaling mechanisms in recurrent tumors that include AR and p160 cofactor overexpression and ligand-independent activation of the AR. The natural androgen decline in the aging male (ADAM) occurs after the age of 40 years, which coincides with the dramatic increase in the prostate cancer incidence in all population groups.

The viability of the following hypothesis is being evaluated: *Prostate cancer progression to androgen independence with age is a consequence of ADAM. ADAM, in turn, selects for prostate cancer cell clones with hypersensitive AR signaling capabilities due to AR amplification, changes in cofactor expression profiles or non-steroidal activation of the AR.* A novel outcome of this work may be the realization that inappropriate AR signaling contributes to PCa risk with age and that the AR is a legitimate target not only for prostate cancer treatments, but also possibly for prevention strategies via hormone replacement with advancing age.

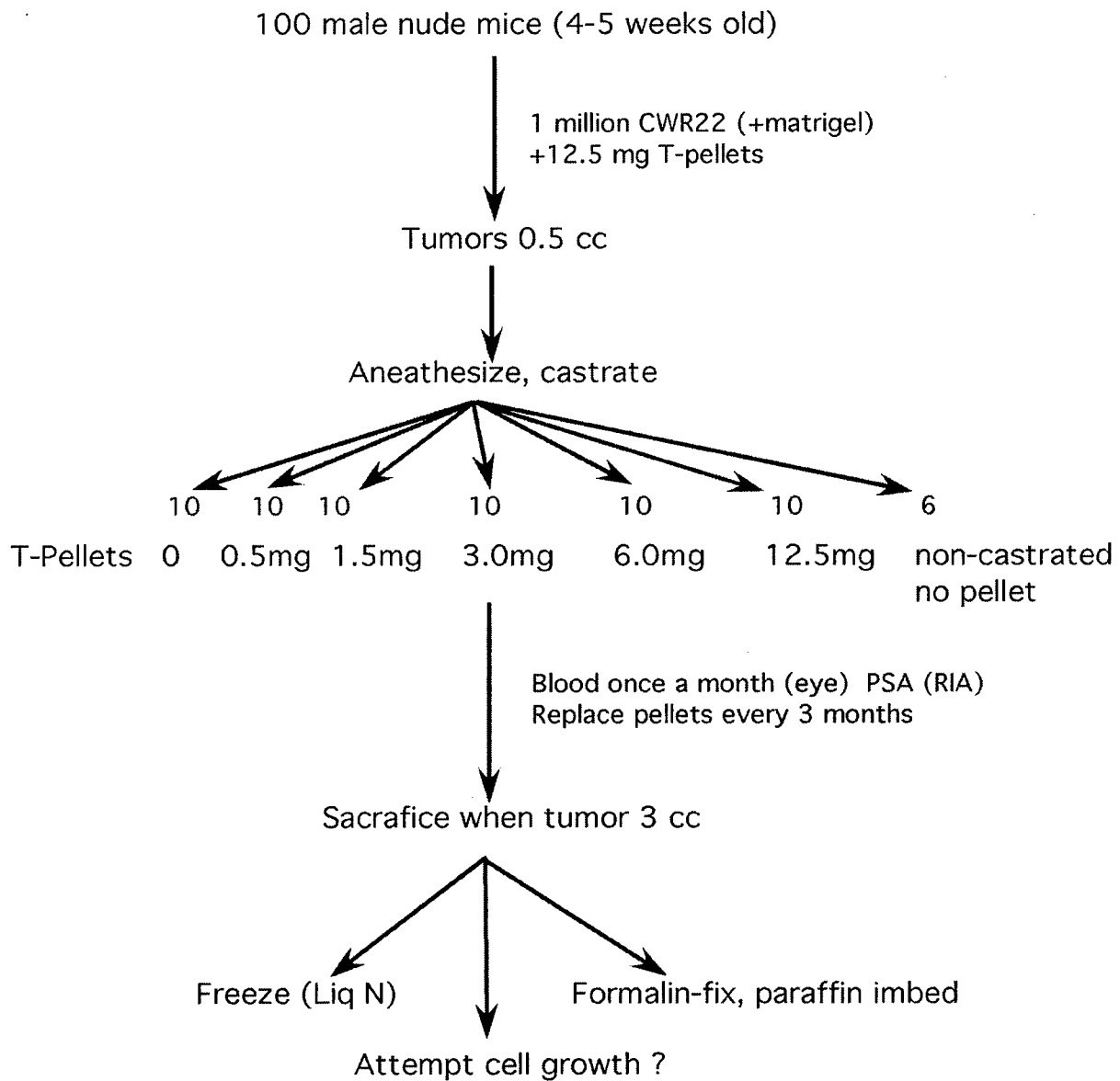
## **Body:**

A single hypothesis is being evaluated (see above) using a mouse xenograft model growing human prostate cancer cells CWR22 cells in nude mice according to the protocol outlined in figure 1 (page 5). The experiment was completed and now awaits analysis of material collected as outlined in the original application (IHC for AR, HER-2 and p160 coactivators and RT-PCR of selected genes including MAPK).

While the above-mentioned experiment was ongoing (it took several months to complete), we did several pilot analyses (in conjunction with other funded work) of AR signaling and made three interesting observations: (i) We defined a novel protein-protein interaction mechanism within the AR (Shen et al, 2005, reference included). (ii) We defined the role of protein kinase A pathway and cAMP responsive element – binding protein in androgen receptor-mediated transcription at the prostate-specific antigen locus (Kim et al 2005, reference included) and (iii) We discovered that AR-dependent PSA expression in androgen-independent prostate cancer cells does not involve AR occupancy of the PSA locus (Jia & Coetzee 2005, reference included). Two review articles were also written partially funded by the present grant (Prescott & Coetzee in press; Shen & Coetzee in press, both included).

Figure 1:

## ADAM protocol



**Key Research Accomplishments:**

1. The animal experiment was completed, now awaiting analyses.
2. AR signaling details were better defined (three articles) partially funded by the present grant.
3. AR signaling was reviewed in two review articles partially funded by the present grant.

**Reportable Outcomes:**

None so-far.

**Conclusions:**

None so-far

**References:**

(All included in the Appendices)

Shen, H.C., Buchanan, G., Butler, L.M., Prescott, J., Henderson, M., Tilley, W.D., Coetzee, G.A.: GRIP1 mediates the interaction between the amino- and carboxyl-termini of the androgen receptor. *Biol. Chem.* 386, 69-74 (2005).

Kim, J., Jia, L., Stallcup, M.R., Coetzee, G.A.: The role of protein kinase A pathway and cAMP responsive element-binding protein in androgen receptor-mediated transcription at the prostate-specific antigen locus. *J. Mol. Endo.* 34, 107-118 (2005).

Jia, L., Coetzee, G.A.: AR-dependent PSA expression in androgen-independent prostate cancer does not involve AR occupancy of the PSA locus. *Can. Res.* In press (2005).

Shen, H.C., Coetzee, G.A.: The Androgen Receptor: Unlocking the secrets of its unique transactivation domain. In Vitamins and Hormones, vol 71 (Academic Press), (2005).

Prescott, J. Coetzee, G.A.: Molecular Chaperones throughout the life cycle of the Androgen Receptor. Cancer Letters (in press) (2005).

**Appendices:**

The five articles referred to above are included.

Short Communication

## GRIP1 mediates the interaction between the amino- and carboxyl-termini of the androgen receptor

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### Abstract

The androgen receptor (AR) mediates transactivation of target genes by acting as a dimer in which its amino-terminal domain (AR-NTD) interacts with its carboxyl-terminal, ligand-binding domain (AR-LBD) (N/C interaction). Here we assessed if and how AR N/C interaction relates to AR transactivation activity and how the p160 coactivator GRIP1 participates in both processes. The concentration of dihydrotestosterone needed for half-maximal N/C interaction was approximately 10-fold higher than for half-maximal transactivation, indicating a disparity between the two processes. Although a mutation of an LXXLL-like motif, <sup>23</sup>FQNLF<sup>27</sup> → <sup>23</sup>FQNAA<sup>27</sup>, in the AR-NTD abolished AR N/C interaction, it could be restored by the co-expression of the coactivator GRIP1. Co-expression of mutated forms of GRIP1, possessing alterations known to abolish either of the two AR interaction domains, could not restore AR N/C interaction, suggesting that wild-type GRIP1 normally bridges the two AR domains. Although AR transactivation activity can proceed without AR N/C interaction, we propose that part of the GRIP1 coactivation activity resides in its ability to bind both AR-NTD and -LBD, to stabilize the N/C complex and allow for secondary cofactors to be recruited more efficiently. Our results also indicate that AR N/C interaction enhances but is not necessary for AR transactivation activity.

**Keywords:** androgen receptor; coactivator; dimerization; domain interactions; transactivation; transcription.

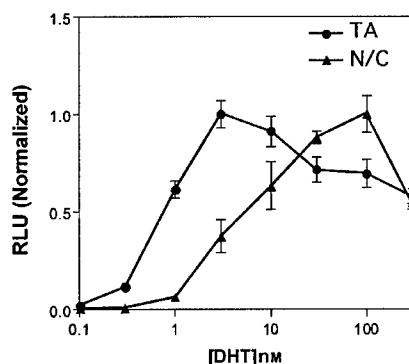
The androgen receptor (AR) has three well-defined modular protein domains: an amino-terminal domain (NTD) involved principally in transcriptional activation, a DNA-

binding domain (DBD) that is required for interaction with specific gene sequences, and a carboxyl-terminal ligand-binding domain (LBD) that mediates the specific interaction between the AR and its cognate ligands (Germann, 2002). Following ligand binding, the LBD undergoes an ordered structural rearrangement that results in the formation of a highly conserved protein-protein interaction surface known as activation function 2 (AF2). Early work indicated that the NTD interacts with the LBD in what has been referred to as AR N/C interaction, and that this is facilitated by coactivators (Ikonen et al., 1997). More recently, it was shown that AF2 of most nuclear receptors is occupied by LXXLL-like motifs [known as nuclear receptor (NR) boxes, where L is leucine and X is any amino acid] of coregulatory proteins, of which the p160 family is the most widely characterized (Ma et al., 1999). In contrast, AF2 of the AR is predominantly occupied by an LXXLL-like peptide, <sup>23</sup>FQNLF<sup>27</sup> of the AR-NTD (He and Wilson, 2002). This interaction is thought to mediate AR homo-dimerization and to be important for AR function *in vivo*. Of essential importance are the following points: (i) AR-AF2 interacts specifically and with greater avidity with this single <sup>23</sup>FQNLF<sup>27</sup> of the AR-NTD than with the NR boxes of p160 coactivators; and (ii) AR N/C interaction inhibits p160 coactivator recruitment to the AR-AF2 in the LBD (He et al., 2001).

We have previously shown through glutathione S-transferase (GST) pull-down assays that a p160 coactivator of AR, GRIP1, interacts with the AR-AF2 region via its LXXLL boxes situated approximately in the middle between the GRIP1 N- and C-termini (Ma et al., 1999), as well as with a C-terminal portion of the AR-NTD via the GRIP1 C-terminal domain (Irvine et al., 2000). This previously published evidence of physical protein/protein interaction between GRIP1 and the separate AR NTD and LBD domains has led us to hypothesize that GRIP1 may bridge AR N/C domains, thereby facilitating or stabilizing AR inter-domain communication, and to propose that the primary consequence of this facilitated bridging is increased AR transactivation activity.

In the present study we assessed whether AR N/C interaction and transactivation of target genes are mechanistically linked and how p160 coactivators participate in the two processes. Both processes are dependent on androgen (dihydrotestosterone, DHT), but to different extents (Figure 1). Concentrations of DHT necessary to yield half-maximal activity were approximately 1 and 10 nM DHT for transactivation activity and N/C interaction, respectively, indicating a disparity between the two processes. For example, at 1 nM DHT, transactivation activity proceeded at approximately half-maximal rates, while N/C interaction was barely detectable (Figure 1),





**Figure 1** DHT dose-response curves of AR transactivation and N/C interaction.

COS7 cells ( $1 \times 10^4$  cells/3-mm well) were transfected with DNA-expressing AR constructs and luciferase reporters. Transfected cells were incubated in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped FBS in the absence or presence of varying concentrations of DHT (0.1, 0.3, 1, 3, 10, 30, 100, and 300 nM). The data represent normalized RLU values, with the maximal activity of each curve set at 1.0. Results of the transactivation experiment represent the mean ( $\pm$ SEM) of 10 individually transfected wells, whereas the results of the N/C interaction experiment represent the mean ( $\pm$ SEM) of 5 individually transfected wells.

**Methods:** Transactivation of the full-length wild-type AR (pcDNA AR WT) activity was assayed using the AR responsive probasin-luc reporter. Full-length AR consisting of amino acids 1–919 was cloned into pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA, USA) as previously published (Irvine et al., 2000). Probasin-luc (ARR3-tk-luc/tk81-PB3) is a mammalian expression vector that contains firefly luciferase linked to three copies of the androgen-responsive minimal rat probasin promoter (–244 to –96) ligated in tandem to the thymidine kinase (tk) enhancer element (Snoek et al., 1998; Kasper et al., 1999) provided by Dr. R.J. Matusik (The Vanderbilt Prostate Cancer Center, Nashville, TN, USA). N/C interaction of the wild-type AR NTD 1–538 VP16-AD fusion vector (VP16 AR-NTD WT) with the AR LBD 644–919 GAL4-DBD fusion vector (GAL4 AR-LBD) was assayed by measuring GAL4-responsive luciferase reporter (pGK1) as relative light units (RLU), using the Mammalian Matchmaker Two-Hybrid Assay Kit (Clontech, Palo Alto, CA, USA). COS7 cells (ATCC, Manassas, VA, USA) were maintained in Dubelco's modified Eagle medium (DMEM) hi-glucose media (USC/Norris Cancer Center Microchemical Core facility) that was supplemented with 5% (v/v) heat-inactivated fetal bovine serum (FBS) and used between passages 7 and 12. Cells were plated in 96-well plates at a density of  $2 \times 10^4$  cells/well and incubated for 24 h in DMEM. Subsequent transient transfection of plasmids was conducted using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's suggested protocol. After transfection, cells were grown in Opti-MEM I reduced serum medium (Invitrogen) in the absence or presence of DHT (Sigma Chemical Co, St. Louis, MO, USA) for 24 h in the case of the mammalian two-hybrid assay, or 36 h in the case of the full-length AR transactivation assay. After incubation cells were lysed with Passive Lysis Buffer (Promega, Madison, WI, USA) and extracts assayed for luciferase activity using the Promega Luciferase Assay System measured on a MLX Microtiter Plate Luminometer (Dynex, Chantilly, VA, USA).

providing evidence that the former can proceed without the latter. One caveat when comparing the data from the two assay systems is that the two-hybrid assay design used to measure N/C interaction may exclude effects of full-length receptor dimerization on DHT binding stability. Thus, while the data reported here represent the isolation

of the N/C interaction event from the overall AR transactivation, more insight into this comparison will come with a better understanding of the role of N/C interaction in AR activation. The data are independently supported by the findings of the Brinkmann and Trapman group (Doesburg et al., 1997) who reached the same conclusions regarding a 10-fold disparity in DHT response of AR N/C interaction versus transactivation.

To understand better the mechanism(s) of AR N/C interaction, the role played by coactivators, and how different molecular interactions participate in AR transactivation activity, we next investigated interdomain interactions of both wild-type AR and a mutant AR that contains alterations known to affect the N/C interaction. When both wild-type AR-NTD and AR-LBD domains were co-expressed in cells in a mammalian two-hybrid assay, treatment with DHT resulted in strong interaction not explicable by activity of either fusion protein expressed with irrelevant partners (Figure 2A). This interaction was enhanced nearly 10-fold when wild-type GRIP1 was co-expressed, suggesting that GRIP1 may act to stabilize the AR-NTD and -LBD interaction, possibly via physically bridging the two AR domains. Recruitment of GRIP1 to GAL4 AR-LBD in the absence of an interacting AR-NTD resulted in near-negligible activity (Figure 2A). When AR-NTD containing the alteration  $^{23}\text{FQNLF} \rightarrow ^{23}\text{FQNAA}$  (VP16 AR-NTD FQNAA) was co-expressed with wild-type AR-LBD, treatment with DHT alone resulted in an N/C interaction that was also only marginally above the no-DHT background level, suggesting that the FQNLF motif is a key interaction surface for DHT-driven AR N/C interaction. However, DHT-dependent N/C interaction involving the  $^{23}\text{FQNAA}$  mutant was dramatically restored when wild-type GRIP1 was co-expressed in the assay, suggesting that the enhancing effect of GRIP1 on N/C interaction could still operate in the presence of the altered AR FQNLF motif. The restored N/C interaction by GRIP1 cannot be attributed to artifacts of intrinsic GRIP1 coactivation activity, as GRIP1 recruitment to GAL4 AR-LBD alone did not result in appreciable activity. Therefore, the recovery of  $^{23}\text{FQNAA}$  N/C interaction by GRIP1 is evidence that enhancement of the AR N/C interaction by GRIP1 is likely due to binding surface(s) in the AR-NTD other than the FQNLF motif.

To explore further the nature of the GRIP1 enhancement of AR N/C interaction, we reasoned that GRIP1 mutants lacking the ability to bind either the AR-NTD or AR-LBD would fail to properly function as a bridge between the domains, and would thus fail to enhance N/C interaction beyond that induced by DHT. We have previously published evidence demonstrating the physical interaction between two independent subdomains of GRIP1 and AR-LBD and AR-NTD through the use of mammalian two-hybrid assays, GST pull-down assays and mutational analyses (Ma et al., 1999; Irvine et al., 2000). To address the implications of these findings in the present experiments, we utilized two GRIP1 mutant constructs, each with impaired binding to one of the AR subdomains. The first contains a truncation that eliminates the C-terminal 341 amino acids [GRIP1 (1–1121)], thus losing the NID<sub>AF1</sub> region previously shown to be nec-



plausible that the increased AR N/C interaction activity caused by GRIP1 in the mammalian two-hybrid assay involving wild-type AR fragments results from three mechanisms: (i) increased N/C interaction through GRIP1 bridging; (ii) increased recruitment of GRIP1 to the transcription complex; and (iii) stabilization of the AR-NTD fusion proteins by GRIP1.

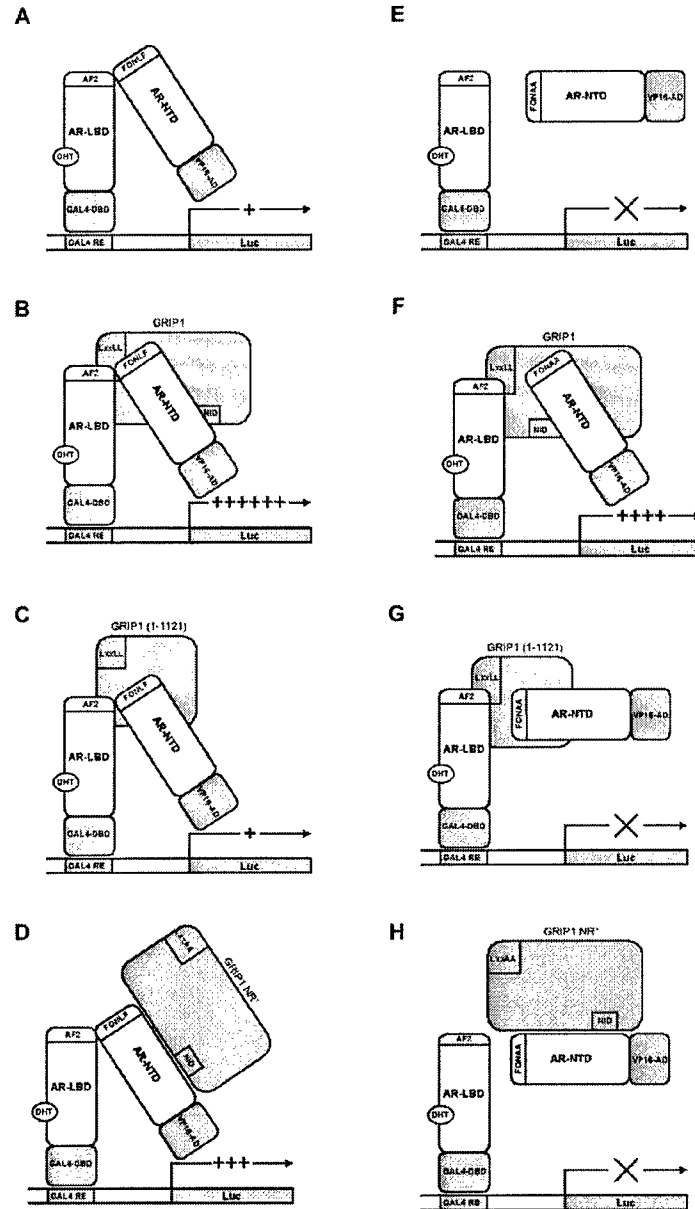
Within the same assay conditions, results involving the AR-NTD FQNAA mutant were dramatically different (Figure 2A). While wild-type GRIP1 rescued the inhibited AR N/C interaction of the AR-NTD FQNAA mutant as previously noted, the two GRIP1 mutants were largely unable to do so, indicating that both AR-NTD and -LBD interacting domains of GRIP1 are necessary to achieve the putative bridging activity. Once again, the small apparent induction of AR N/C interaction observed in response to the co-expression of GRIP1 NR\* mutant is likely due to the activity of GRIP1 NR\* recruited to the small amount of AR-NTD engaged in the marginal N/C interaction involving the AR-NTD FQNAA mutant. It is clear that substantially lower AR N/C interaction activity occurred between AR-NTD FQNAA and AR-LBD in the presence of either of the GRIP1 mutants compared to the activity of wild-type AR-NTD.

We have schematically depicted the different molecular interactions involved in the AR N/C mammalian two-hybrid assay (Figure 3). Figure 3A–D illustrates the interactions involving wild-type AR-NTD, whereas Figure 3E–H, the interactions involving the AR-NTD <sup>23</sup>FQNAA<sup>27</sup> mutant. We propose that wild-type GRIP1 stabilizes wild-type AR N/C interactions, thereby mediating coactivation (Figure 3B). However, mutant GRIP1 (1-1121) is unable to do so (Figure 3C), indicating that the LXXLL boxes of GRIP1 are unable to compete effectively against <sup>23</sup>FQNLF<sup>27</sup> of the AR-NTD for AR-LBD binding. On the other hand, mutant GRIP1 (GRIP1 NR\*) that is able to bind to the AR-NTD, but not to the AR-LBD, can presumably still mediate coactivation activity in the assay, albeit not to the level of wild-type GRIP1 (Figure 3D). The AR-

NTD <sup>23</sup>FQNAA<sup>27</sup> mutant (which is unable to mediate AR N/C interaction; see Figure 3E) can be bridged to AR-LBD by wild-type GRIP1, yielding high activity (Figure 3F), but not to the same extent with GRIP1 (1-1121) (Figure 3G), or GRIP1 NR\* (Figure 3H), again probably due to the GRIP1 mutants lacking either one of their AR-interacting surfaces. Our results therefore indicate that AR N/C interaction can occur directly via the involvement of AR-NTD <sup>23</sup>FQNLF<sup>27</sup> or indirectly via GRIP1 bridging.

We next examined the effects of GRIP1 and its mutants on wild-type and <sup>23</sup>FQNAA<sup>27</sup> mutant AR transactivation activity. Co-expressed wild-type GRIP1 resulted in an approximately three-fold increase in DHT-dependent receptor activity (Figure 2B). When either of the two mutant GRIP1 constructs was co-expressed in place of wild-type GRIP1, the receptor transactivation activity was still enhanced over that of DHT stimulation alone, albeit modestly compared to wild-type GRIP1. The situation with AR FQNAA mutant was very similar and, importantly, substantial transactivation activity was observed for this AR mutant, even in the presence of mutant GRIP1 molecules. Most notably, these same GRIP1 mutants were unable to rescue N/C interaction activity between AR-NTD FQNAA and AR-LBD, as previously discussed (Figure 2A). Whereas coactivation of the FQNAA mutant AR by wild-type p160 coactivators was previously reported by the Wilson group (He et al., 2002), the present work demonstrates that this coactivator response may occur mechanistically through the restoration of impaired N/C interaction by coactivator molecules bridging AR domains. However, given that the AR FQNAA mutant maintains substantial transactivational competence, the rescue of its nearly completely impaired N/C interaction should be regarded as a mechanism that further contributes to, but is not critical for, transactivation. Thus, the disparity between levels of AR N/C interaction and AR transactivation activity reported here indicates that AR N/C interaction is not necessary for AR transactivation activity.

**Figure 2** Methods: Mutations in the AR were constructed using the protocol published in the Stratagene Quick-Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). pcDNA AR FQNAA codes for full-length AR with the <sup>23</sup>FQNAA<sup>27</sup> mutation. AR-NTD FQNAA codes for AR transactivation domain amino acids 1–538 containing the <sup>23</sup>FQNAA<sup>27</sup> mutation. PSA-luc consists of firefly luciferase driven by the androgen-responsive 548-bp PSA promoter region (–541 to +7) preceded by 1450 bp of the PSA enhancer region (–5322 to –3873) (Bristol-Myers Squibb, Princeton, NJ, USA). Three GRIP1 expression constructs (a gift from the laboratory of Dr. Michael Stallcup) consisted of PSG5 GRIP1 coding for full-length GRIP1 amino acids 1–1462, PSG5 GRIP1 (1-1121) coding for a truncated form of the protein lacking activation domain 2, and PSG5 GRIP1 NR\* coding for the full-length protein having the second and third LXXLL motifs in the NR-box domains mutated to LXXAA. In all experiments we balanced molar amounts of expression vectors with their empty counterparts as controls and balanced the nanogram amounts of total DNA with pCAT-basic, a eukaryotic promoter-less vector. Under these conditions, experimental and control situations contained identical concentrations of total DNA per transfection (i.e., ng/transfection) and an identical copy number of promoter elements (i.e., molar amounts). For Western immunoblot analyses COS7 cells were plated in 60-mm dishes at a density of  $6 \times 10^5$  cells/dish and grown in DMEM hi-glucose for 24 h prior to DNA transfection using LipofectAMINE 2000 reagent. Treatment with DHT and subsequent incubation times paralleled the 96-well plate protocols described above and in the legend of Figure 1. Cells were then harvested in 100  $\mu$ l of RIPA buffer (10 mM sodium phosphate, 2 mM EDTA, 150 mM NaCl, 50 mM NaF, 0.1% SDS, 1% IGEPAL CA-630, 1% sodium deoxycholate, 0.2 mM Na<sub>2</sub>VO<sub>4</sub>, pH 7.2) that contained a cocktail of mammalian protease inhibitors (Sigma-Aldrich). Equal volumes of each extract were analyzed by SDS-PAGE. Proteins were transferred to Hybond-P membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and probed with anti-AR (N20) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-GAL4-DBD monoclonal antibody (Clontech), anti-VP16-AD polyclonal antibody (Clontech) and anti-actin antibody (Santa Cruz Biotechnology). HRP-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) and goat-anti mouse HRP-conjugated antibody (BD Biosciences, San Jose, CA, USA) were used as secondary antibodies. Detection was carried out using Western Blotting Luminol Reagent (Santa Cruz Biotechnology) and an enhanced chemiluminescence Western blotting system (Amersham Pharmacia Biotech) according to the manufacturers' protocols. Coomassie Brilliant Blue staining of the gel showed no significant differences in protein loading. Chemiluminescent images were analyzed by a Fluor-S Max Multimager Quantification System (Bio-Rad, Hercules, CA, USA).



**Figure 3** Molecular interactions involved in the AR N/C mammalian two-hybrid assay.

The DHT-induced VP16 AR-NTD/GAL4 AR-LBD interactions driving expression of luciferase reporter in the absence or presence of GRIP1 coactivator are depicted schematically. The relative strength of luciferase activity detected is represented symbolically using plus symbols (+) to indicate activity detected above background levels, or crosshatch (X) indicating little to no luciferase activity detected. All models depict scenarios following DHT binding to AR-LBD. Models A, B, C, and D depict actions of wild-type AR-NTD <sup>23</sup>FQNLF<sup>27</sup> in the assay. Models E, F, G, and H depict actions of mutant AR-NTD <sup>23</sup>FQNAA<sup>27</sup> in the assay. (A,E) AR-LBD interacts through the AF2 motif with the <sup>23</sup>FQNLF<sup>27</sup> motif of wild-type AR-NTD. This interaction is lost by mutant AR-NTD <sup>23</sup>FQNAA<sup>27</sup>. (B,F) The presence of wild-type GRIP1 enhances reporter activity through bridging of AR N/C interaction. Bridging is mediated by GRIP1 LXXLL and NID<sub>AF1</sub> motifs binding to respective domains of AR. GRIP1 bridging results in rescue of the impaired AR-NTD <sup>23</sup>FQNAA<sup>27</sup>/AR-LBD interaction. (C,G) Truncated GRIP1 (1-1121) fails to bind AR-NTD, cannot bridge across AR domains, and thus has no impact on DHT-induced AR N/C interaction. (D,H) Mutant GRIP1 NR\* fails to bind AR-LBD. Marginal GRIP1 NR\* recruitment to the transactivation complex may occur through preserved binding to AR-NTD, resulting in modest enhancement of reporter activity in the context of wild-type AR N/C interaction.

In summary, we have demonstrated that GRIP1 acts as a bridge between AR-NTD and -LBD. Furthermore, a disparity between AR N/C interaction and AR transactivation activity was evident, since the two processes had different DHT dependence and GRIP1 mutants were unable to rescue the impaired AR N/C interaction experi-

enced by an AR-NTD mutant, while the transactivation activity of this AR mutant remained largely intact. We therefore conclude that AR N/C interaction, as mediated either directly by FQNLF/AF2 interaction or indirectly by GRIP1 bridging, is involved in the enhancement of, but is not necessary for, AR transactivation activity. The data

presented here indicate that the relatively large and unstructured AR-NTD is pleiotropic in its transcriptional regulation, and strongly suggest that this flexibility may be utilized in response to changing cellular signaling environments during normal cell growth and differentiation, as well as abnormal situations such as during the progression of prostate cancer.

## Acknowledgments

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# The role of protein kinase A pathway and cAMP responsive element-binding protein in androgen receptor-mediated transcription at the prostate-specific antigen locus

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## Abstract

Androgen-independent prostate cancer is a lethal form of the disease that is marked by metastasis and rapid proliferation in its final stages. As no effective therapy for this aggressive tumor currently exists, it is imperative to elucidate and target the mechanisms involved in the progression to androgen independence. Accumulating evidence indicates that aberrant activation of androgen receptor (AR) via signal transduction pathways, AR gene mutation and/or amplification, and/or coregulator alterations may contribute to the progression of prostate cancer. In the present study, the effects of protein kinase A (PKA) signaling and its downstream factors on AR activity at the prostate-specific antigen (PSA) gene were tested. Activation of PKA by forskolin resulted in enhanced androgen-induced expression of the PSA gene, an effect that was blocked by the AR antagonist, bicalutamide. Interestingly, when either p300 or CBP was overexpressed, PKA activation was sufficient to stimulate PSA promoter-driven transcription in the absence of androgen, which was not inhibited by bicalutamide. PKA activation did not significantly alter AR protein levels but significantly increased the phosphorylated form of its downstream effector, cAMP responsive element-binding protein (CREB) in the presence of androgen. Furthermore, chromatin immunoprecipitation showed that the combination of androgen and forskolin increased phosphorylated CREB occupancy, which was accompanied by histone acetylation, at the putative cAMP responsive element located in the 5' upstream regulatory region of the PSA gene. Remarkably, mammalian two-hybrid assay indicated that p300/CBP may bridge the interaction between AR and CREB, suggesting a novel enhanceosomal cooperation. These results demonstrate an intriguing interplay between a signal transduction pathway, coactivator overexpression and AR signaling as a possible combined mechanism of progression to androgen-independent prostate cancer.

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## Introduction

Untreated prostate cancer progresses from androgen-dependent tumors that respond favorably to clinical therapies to recurrent, metastatic, androgen-independent tumors that are invariably fatal. Even at the latter, hormone-refractory stage, the tumor growth is dependent on a functional androgen receptor (AR) signaling pathway, as most androgen-independent prostate tumors continue to express AR and AR-regulated genes, such as prostate-specific antigen (PSA) (Buchanan *et al.* 2001). This evidence suggests that a critical process in tumor recurrence and progression may involve some compensatory regulation of AR activity in the low-androgen environment. Although the exact molecular basis for the progression to androgen-independent prostate cancer has yet to be defined, it is becoming increasingly clear that more than one specific mechanism may be involved. The proposed means of subverting growth control include AR gene mutation

and amplification, coregulator alterations and androgen-independent activation of AR via various signal transduction pathways (Feldman & Feldman 2001). These mechanisms are not mutually exclusive but may in fact constitute compensatory measures that cooperate to establish androgen independence (Weber & Gioeli 2004).

Recent attention has focused on the hypothesis that the aberrant activation of AR through various signal transduction pathways plays an important role in tumor progression. Numerous studies indicate that AR signaling is affected by a diverse array of cytokines and growth factors, such as insulin-like growth factor 1 (IGF-1), keratinocyte growth factor (KGF), epidermal growth factor (EGF), interleukin 6 (IL-6) and forskolin, which act through a web of complex signal cascades, such as mitogen-activated protein kinase (MAPK), janus-activated kinase (JAK), signal transducers and activators of transcription (STAT), and protein kinase A (PKA) (Culig *et al.* 1994, Nazareth & Weigel 1996,

Reinikainen *et al.* 1996, Sadar *et al.* 1999, Ueda *et al.* 2002, Jia *et al.* 2004). Of these pathways, PKA, as activated by forskolin, is particularly interesting because of its possible ability to phosphorylate AR *in vivo* and stimulate the expression of the AR-regulated gene, PSA, in the absence of androgen (Sadar *et al.* 1999, Gioeli *et al.* 2002). Taken together, these observations suggest that PKA may modulate AR function, in part, by activating AR through phosphorylation in the absence of androgen. However, site-directed mutagenesis of AR phosphorylation sites has shown that the transcriptional activity of AR may not be affected by the post-translational modification (Gioeli *et al.* 2002). Thus, it is plausible that other mechanisms downstream of the PKA pathway are involved in the activation of AR-regulated genes.

One such mechanism that is downstream of the PKA pathway is the phosphorylation of cAMP responsive element-binding protein (CREB). CREB is a conceivable candidate for mediating PKA effects on AR-regulated transcription due to the presence of a putative cAMP responsive site (CRE) at the 5' upstream regulatory region of the PSA gene (Sadar *et al.* 1999). Although no reports have yet confirmed this phenomenon, it is a striking association in view of the PKA-induced transcription of PSA and the mechanism of CREB activity. PKA stimulation by cAMP, which is synthesized by forskolin-activated adenylyl cyclase, leads to the phosphorylation of CREB at serine 133 (Ser 133), located within a transcriptionally critical region called kinase-inducible domain (KID) (Mayr & Montminy 2001). Phosphorylation of Ser 133 promotes binding of CREB to CRE and recruitment of the coactivator histone acetylase (HAT) paralogs CREB-binding protein (CBP) and p300 (Shaywitz & Greenberg 1999). The recruitment of CBP/p300 may specifically enhance the transcriptional potential of CREB by acetylating histones around the CRE and/or bridging the interaction between CREB and components of the basal transcription machinery (Nakajima *et al.* 1996, Korzus *et al.* 1998).

Interestingly, the physical bridging function of CBP/p300 and their HAT activities are both fundamentally important features that are also shared in the transcription activation modulated by nuclear hormone receptors, including the AR. Compelling studies suggest that, in the specific case of PSA gene transcription, CBP/p300 may not only bridge the interaction between AR and the basal transcription apparatus but also mediate the contacts between AR/coactivator complexes bound to the promoter and enhancer regions, thus forming 'enhanceosomes' (Shang *et al.* 2002, Louie *et al.* 2003). This theory of communication between 5' regulatory elements is especially intriguing because of its implication that CBP/p300 could possibly mediate interactions between different transcription factors, such

as AR and CREB, that share coactivators in common. Furthermore, another inference of the theory is that coactivators may play a crucial role in coordinating the increased transcriptional activity of AR in low-androgen environments by assembling stable, higher-order complexes. Indeed, transcriptional synergy among nuclear receptor coactivators via ternary complex formation has been documented (Lee *et al.* 2002). This also suggests that increased expression of coactivators may be an important mechanism that could compensate for the androgen-depleted state of advanced prostate cancer.

In the present study, the effects of PKA signaling and its mechanism of action on AR-regulated transcription at the PSA gene were investigated. The experiments confirmed that PSA gene expression was enhanced by PKA activation and demonstrated a novel mechanism of CREB and AR cooperation through CBP/p300. The evidence illustrated here presents a possibility of a unique cross-talk mechanism between AR and PKA signaling pathways.

## Materials and methods

### Cell culture and reagents

The human prostate cancer cell line LNCaP was obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in RPMI 1640 (Invitrogen, Grand Island, NY, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gemini Bioproducts, Woodland, CA, USA); it was used between passages 19 to 24. Cos 7 cells were also obtained from the ATCC and grown in OPTI-MEM (Invitrogen) supplemented with 5% (v/v) heat-inactivated FBS; they were used between passages 12 to 16. 5 $\alpha$ -Dihydrotestosterone (DHT) was purchased from Sigma-Aldrich (St Louis, MO, USA). H-89 was purchased from Calbiochem (San Diego, CA, USA). Bicalutamide was obtained from Astra Zeneca. Forskolin was purchased from Sigma-Aldrich.

### Transient transfection and luciferase detection

LNCaP cells ( $5 \times 10^4$  cells/well) in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped FBS (Gemini) were plated in 96-well plates for 3 days. Cells were then transfected with reporter PSA-luc (100 ng/well; pGL3\_PSA540), pCMV-300 or pCMV-CBP (50 ng/well; Dr T-P Yao, Duke University, Durham, NC, USA), and/or pCAT-basic (50 ng/well; Promega), a negative control to balance out the total amount of DNA, using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. pGL3-PSA540-enhancer

(PSA-luc) is a mammalian expression vector that contains firefly luciferase linked to the androgen-responsive 548 bp PSA promoter region (−541 to +7) preceded by 1450 bp of the PSA enhancer region (−5322 to −3873) provided by Bristol-Myers Squibb (Princeton, NJ, USA). After transfection, cells were grown in phenol red-free RPMI 1640 containing 0.5% charcoal/dextran-stripped FBS with DHT, forskolin, bicalutamide and/or H-89 as indicated. After additional 30-h incubation, the cells were lysed with the passive lysis buffer (Promega). The extracts were assayed for luciferase activity with the Promega kit, according to the manufacturer's protocol, and measured on a Dynex MLX Microtiter Plate Luminometer (Chantilly, VA, USA). Relative luciferase units (RLU) are shown as the means  $\pm$  S.D. of quadruplicate wells. Total protein concentrations of the extracts were assayed with the Bio-Rad Protein Assay Kit (Hercules, CA, USA), according to the manufacturer's protocol, and 600 nm absorbance was measured on a Molecular Devices EMax Microplate Reader (Sunnyvale, CA, USA). No significant differences in total protein concentrations were observed among the different wells within a given experiment.

#### Chromatin immunoprecipitation (ChIP) assays

LNCaP cells ( $5 \times 10^6$  cells/150 mm dish) were cultured in phenol red-free RPMI 1640 supplemented with 5% charcoal/dextran-stripped FBS (Gemini) for 3 days. Cells were treated with DHT and/or forskolin for 2 h, cross-linked by adding formaldehyde (1%) directly to the culture medium, and incubated at room temperature for 10 min. The cells were washed twice with ice-cold PBS and harvested by scraping and centrifugation at 3000 *g* for 5 min. The cell pellets were resuspended in 0.5 ml lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.0, with 1  $\times$  complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA)) and incubated for 20 min on ice. The cell lysates were sonicated at setting 4 on a Branson Sonifier Cell Disruptor 185 for 10 s (Fisher Scientific, Los Angeles, CA, USA). The sonication was repeated five times (with 1-min incubations on ice between sonications), and insoluble materials were removed by centrifugation at 15 500 *g* for 10 min. For each immunoprecipitation, 100  $\mu$ l supernatant containing soluble chromatin was diluted 10-fold in dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.0), 167 mM NaCl and 1  $\times$  protease inhibitor cocktail). After preclearing with 75  $\mu$ l of protein G-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ, USA) at 4 °C for 1 h, the supernatant was immunoprecipitated by incubating at 4 °C overnight with 25  $\mu$ l anti-AR (N20, Santa Cruz Biotechnology, Santa Cruz, CA, USA), 10  $\mu$ l anti-CREB, 10  $\mu$ l anti-phospho CREB, 5  $\mu$ l anti-dimethyl H3-K4 and 5  $\mu$ l anti-Ach3 (Upstate

Biotechnology, Lake Placid, NY, USA). Immune complexes were obtained by incubating with 50  $\mu$ l protein G-Sepharose at 4 °C for 1 h. Immunoprecipitates were sequentially washed for 5 min each in low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0) and 150 mM NaCl), high-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0) and 500 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1% IGEPAL CA 630, 1% deoxycholate, 1 mM EDTA and 10 mM Tris-HCl (pH 8.0)) and TE buffer (twice). Washed beads were extracted with 250  $\mu$ l elution buffer (1% SDS and 100 mM NaHCO<sub>3</sub>) twice, the elution was combined, and the protein-DNA cross-linking was reversed by incubation at 65 °C overnight. Each sample was treated with 20  $\mu$ g proteinase K (Gibco BRL, Grand Island, NY, USA) in proteinase K buffer (50 mM Tris-HCl (pH 6.5) and 10 mM EDTA) at 45 °C for 1 h. DNA was purified by phenol/chloroform extraction, precipitated by ethanol with glycogen, and resuspended in 100  $\mu$ l H<sub>2</sub>O. One percent of total soluble chromatin was processed in parallel without immunoprecipitation, and values obtained from this DNA were used as denominators to calculate immunoprecipitated DNA as percentage of input.

#### Real-time PCR of ChIP DNA

Immunoprecipitated DNA was analyzed by quantitative real-time PCR, as described previously (Jia *et al.* 2003). Briefly, triplicate PCRs for each sample were mixed with AmpliTaq Gold PCR master mix (Applied Biosystems, Branchburg, NJ, USA), forward and reverse primers and probe to be analyzed by Bio-Rad iCycler optical system. The primers and probes were as follows: enhancer forward, 5'-GCCTGGATCTGAGAGAGATATCATC-3'; reverse, 5'-ACACCTTTTCTTCTGATTGTTG-3'; CRE forward, 5'-CTGGGCAAGCAATCTGA-3'; reverse, 5'-GTGTGATGCTGGATGATGAGTG-3'; enhancer probe, 5'-6-FAM-TGC AAGGATGCCTGCTTTACAAACATCC-BHQ-1-3'; and CRE probe, 5'-6-FAM-CATGAAAGATGCCCCAGAGGCCTTG-BHQ-1-3' (Biosearch Technologies, Novato, CA, USA). This assay provides a precise quantitation of target DNA and is based on the principle of fluorophore release from a self-quenching probe; the instrument measures the number of cycles (*C<sub>t</sub>*) required for fluorescence to exceed a set threshold. A standard curve of known target DNA is constructed in parallel, from which the relative amount of target DNA in the sample is calculated. Values are presented as percentage input, which is analyzed at the same time. The precise quantitative nature of this analysis is superior to analyses commonly used by others that rely on semiquantitative end-point assessments of PCR bands on agarose gels.



### Western blot analysis

LNCaP cells ( $6 \times 10^5$  cells/60 mm dish) were plated and grown in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped FBS for 3 days. After treatment with DHT, forskolin and/or H-89 as indicated, cells were harvested in 100  $\mu$ l RIPA buffer (10 mM sodium phosphate, 2 mM EDTA, 150 mM NaCl, 50 mM NaF, 0.1% SDS, 1% IGEPAL CA-630, 1% sodium deoxycholate and 0.2 mM  $\text{Na}_3\text{VO}_4$  (pH 7.2)) that contained a cocktail of mammalian protease inhibitors. Total protein concentration of the protein extract was determined by the Bio-Rad Protein Assay Kit, according to the manufacturer's protocol, and 600 nm absorbance was measured on a Molecular Devices Emax Microplate Reader (Sunnyvale, CA, USA). Equal amounts of each protein extract were analyzed by SDS-PAGE, transferred to Hybond-P membrane (Amersham Pharmacia Biotech), and probed with polyclonal anti-AR (AR-N20), anti-actin (Santa Cruz Biotechnology) and anti-CREB/anti-phospho-CREB (Upstate Biotechnology). HRP-conjugated anti-IgG (Santa Cruz Biotechnology) was used as the secondary antibody. Detection was performed by the enhanced chemiluminescence Western blotting system (Amersham Pharmacia Biotech), according to the manufacturer's protocol. Images are representative of three independent immunoblots and were analyzed by the Fluor-S Max MultiImager Quantification System (Bio-Rad).

### Real-time RT-PCR of RNA

PSA mRNA was analyzed, as described previously, by quantitative RT-PCR. After treatment of the cells with DHT and/or forskolin, total cellular RNA was prepared and treated with RNase-free DNase I by the SV Total RNA Isolation System (Promega). A two-step RT-PCR method was employed, using the TaqMan Gold RT-PCR Kit (Applied Biosystems). In these analyses, the same probe and reverse primer located in exon 4 of the PSA gene were used for both the pre- and mature mRNA determinations (see below). The forward primer is located in intron 3 for pre-mRNA and in exon 3 for mature mRNA determinations. mRNA quantitation was performed by real-time RT-PCR. Thus, the primers and probes were as follows: PSA mRNA forward, 5'-GGCAGCATTGAACCAGAGGAG-3'; PSA reverse, 5'-GCATGAACCTTGGTCACCTTCTG-3'; PSA probe, 5'-6-FAM-ATGACGTGTGTGCGCAAGTTCACCC-BHQ-1-3'; GAPDH forward, 5'-GTC-ATGGGTGTGAACCATGAGA-3'; GAPDH reverse, 5'-GGTCATGAGTCCTTCCACGATAC-3'; and GAPDH probe, 5'-6-FAM-CAGCCTCAAGATCATCAGCAATGCCTC-BHQ-1-3' (Biosearch Technologies). TriPLICATE PCR reactions were conducted. GAPDH mRNA expression was analyzed in each sample in parallel wells.

The results are represented as PSA/GAPDH mRNA ratios. Due to extensive DNase treatment of the RNA preparations (total RNA Isolation System; Promega), no significant genomic DNA contamination was apparent: 1. negative controls lacking reverse transcriptase were normally less than 1% of the experimental values (the value of each sample was adjusted by subtracting these negative values); 2. PCR analyses of promoter sequences gave negligible values.

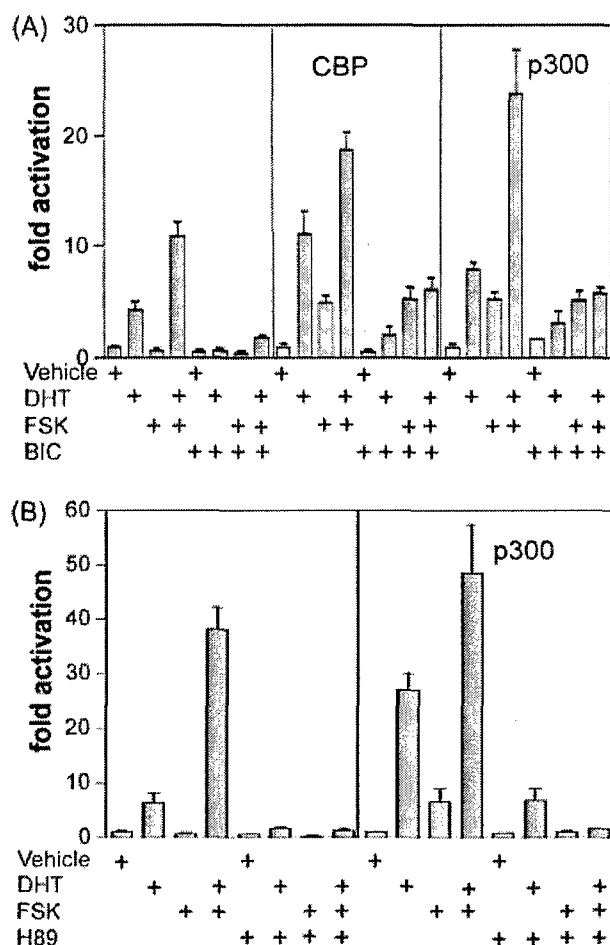
### Mammalian two-hybrid assay

We used reagents from the Clontech Mammalian Matchmaker Two-Hybrid Assay Kit to clone full-length CREB (pCF-CREB kindly provided by M R Montminy of the Salk Institute, La Jolla, CA, USA) into pM vector, and AR NTD and AR LBD into VP16 vector. Cos 7 cells ( $2 \times 10^4$  cells/well) in phenol red-free OPTI-MEM, containing 5% charcoal/dextran-stripped FBS (CSS), were plated in 96-well plates for 24 h. Cells were then transfected with reporter PGK1 (100 ng/well), pM CREB (20 ng/well), VP16 T Ag or VP16 AR NTD (20 ng/well), and GRIP1, p300 or CBP (50 ng/well), using Lipofect AMINE 2000 (Invitrogen), according to the manufacturer's protocol. After 4-h transfection, the lipofection mixture was removed and replaced with 5% CSS media with either ethanol (vehicle), DHT or forskolin as indicated. After 24-h incubation at 37 °C, the medium was removed, and cells were lysed with the passive lysis buffer (Promega). The extracts were assayed for luciferase activity as described above.

## Results

### Forskolin induces CBP/p300-mediated transcription of PSA-driven reporter

Previously, we reported that forskolin enhanced androgen-induced transcription of a PSA-driven reporter gene (Jia *et al.* 2003). In the present study, we sought to explore plausible mechanisms responsible for the observed effect, and considered downstream consequences of PKA activation. Our initial hypothesis was that the forskolin-activated PKA pathway may phosphorylate proteins involved in the AR signaling pathway, including AR and its coactivators. However, a recent study has demonstrated that the phosphorylation of AR does not affect its transcriptional activity (Gioeli *et al.* 2002). Thus, we examined whether coactivators that can be phosphorylated, such as CBP and p300 (Kitabayashi *et al.* 1995, Ait-Si-Ali *et al.* 1998), mediate the increased transcription through PKA signaling. We cotransfected LNCaP cells, which express a functional AR, with a luciferase reporter driven by PSA promoter and enhancer along with, either control, CBP or p300 plasmid (Fig. 1A). As previously described (Jia *et al.* 2003), transcription of



**Figure 1** Forskolin enhances DHT-mediated and induces CBP/p300-mediated PSA transcription. (A) Transient transfection of LNCaP cells with AR-responsive reporter, PSA-luciferase, along with control (pCAT-basic), CBP or p300 expression plasmid. The transfected cells were treated with various combinations of ethanol (vehicle), 10 nM 5 $\alpha$ -dihydrotestosterone (DHT), 50  $\mu$ M forskolin (FSK) and/or 10  $\mu$ M bicalutamide (BIC) as indicated. The values are represented as fold activation over the vehicle control (set as value, 1) in each transfection. (B) Transient transfection of LNCaP cells with PSA-luciferase with either pCAT-basic (as a control) or p300 expression plasmid. The transfected cells were treated with various combinations of vehicle, 10 nM DHT, 50  $\mu$ M FSK and/or 10  $\mu$ M H-89 (specific PKA inhibitor). The values are represented as fold activation over the vehicle control in each transfection. All values are means  $\pm$  S.E.M. of quadruplicate determinations, and the data are representative of three independent experiments.

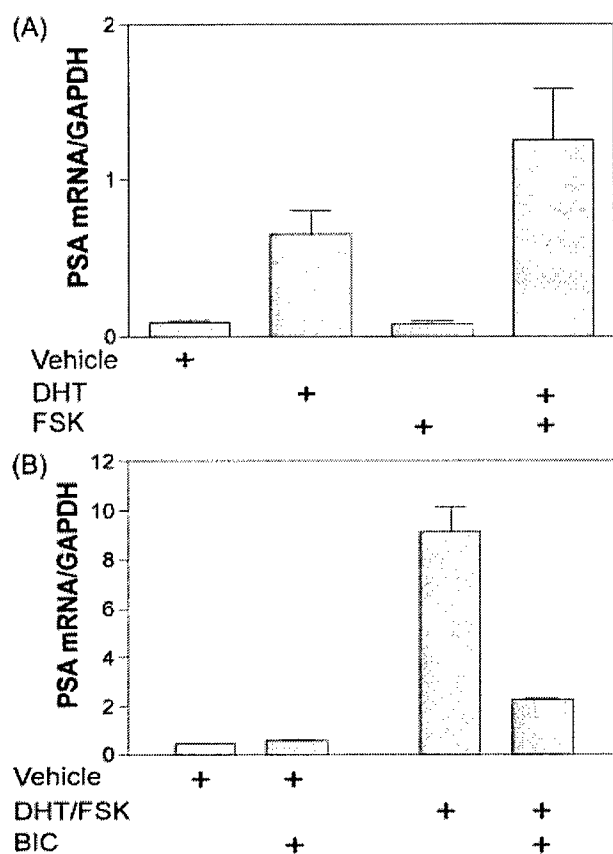
PSA reporter induced by the AR ligand, DHT, was significantly enhanced by forskolin treatment. This effect was inhibited by the addition of the AR antagonist, bicalutamide, suggesting that AR signaling is necessary. Contrary to the earlier report by Sadar *et al.* (1999), we did not detect androgen-independent stimulation of PSA reporter transcription by forskolin treatment alone. Inter-

estingly, however, when we overexpressed CBP or p300, we did observe forskolin-induced transactivation. Moreover, this induction was not inhibited by bicalutamide, indicating that AR does not mediate the androgen-independent stimulation. Moreover, when AR was blocked by bicalutamide, transcription stimulated by DHT and forskolin was reduced to the level of forskolin treatment alone. These results suggested that when CBP or p300 is overexpressed, a mechanism other than AR signaling is stimulated by the PKA pathway, and when combined with AR activity, it produces at least an additive, if not a synergistic, effect on PSA transcription. Next, we tested the effects of PKA pathway suppression with a specific inhibitor, H-89 (Fig. 1B). Surprisingly, when PKA signaling was blocked, both DHT- and DHT/forskolin-induced activities were abolished, indicating that the PKA pathway may be a crucial component of AR signaling in PSA gene transactivation. When p300 was overexpressed, forskolin stimulation was also inhibited by H-89, confirming that PKA signaling is responsible for the CBP/p300-mediated stimulation.

#### PKA activation enhances endogenous PSA gene expression and CREB phosphorylation

Due to the limitations of transient transfection assays, we were cautious in interpreting the transient transfection results; therefore, we sought to investigate the PKA effects *in vivo*. A quantitative analysis of PSA mRNA expression in LNCaP cells yielded similar results to the transient assays (Fig. 2A). Again, in contrast to the findings by Sadar *et al.* (1999), we did not observe an androgen-independent production of PSA mRNA by forskolin treatment alone. PKA activation by forskolin, however, further stimulated DHT-induced PSA mRNA expression. This increased stimulation was significantly reduced but not completely abolished by bicalutamide (Fig. 2B), suggesting that inhibition of AR signaling does not eliminate the PKA component of the stimulation.

Next, we examined whether AR protein levels are affected by PKA activation (Fig. 3A). Addition of forskolin alone or in combination with DHT did not enhance AR expression. Taken together, these data strongly indicate that the enhanced expression of PSA by forskolin is not due to an increased expression or stabilization of AR by PKA signaling. Therefore, considering the results thus far and also the fact that CBP/p300 do not bind to DNA elements directly, we postulated that there may be another transcription factor that mediates both androgen-dependent enhancement and androgen-independent induction of PSA transcription. An immediately apparent candidate was the CREB protein because of its ability to be activated by PKA signaling and its association with CBP/p300. We measured the endogenous expression of CREB in LNCaP cells and determined that the protein level did

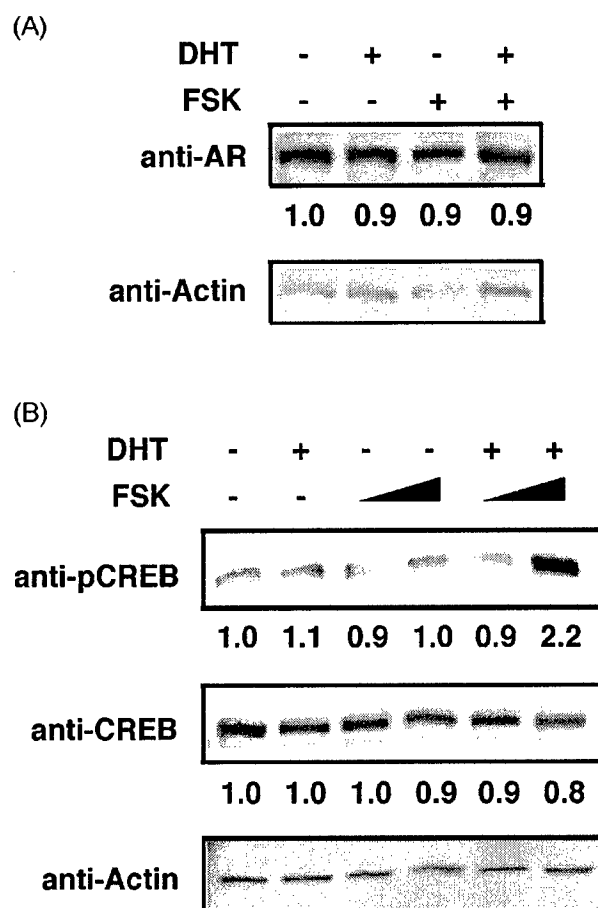


**Figure 2** PKA activation enhances endogenous PSA gene expression. (A) Quantitative RT-PCR of PSA mRNA isolated from LNCaP cells after treatment of vehicle, DHT (ethanol), 10 nM DHT and/or 1  $\mu$ M forskolin (FSK) for 16 h. The values are represented as a ratio of PSA mRNA to GAPDH. (B) PSA mRNA expression induced by DHT and FSK is inhibited by bicalutamide (BIC). LNCaP cells were treated with vehicle, DHT (ethanol), 10 nM DHT and 1  $\mu$ M FSK, and/or 10  $\mu$ M BIC for 16 h. The values are represented as means of ratios of PSA mRNA to GAPDH  $\pm$ S.E.M. of triplicates, and are representative of five independent experiments.

not vary with DHT and/or forskolin exposure (Fig. 3B). However, the level of the phosphorylated form of CREB was significantly elevated by the DHT and forskolin combination. This evidence suggested the interesting possibility of a cross-talk between AR and PKA signaling that synergistically induces CREB activation, which in turn enhances androgen-dependent activation of PSA gene transcription.

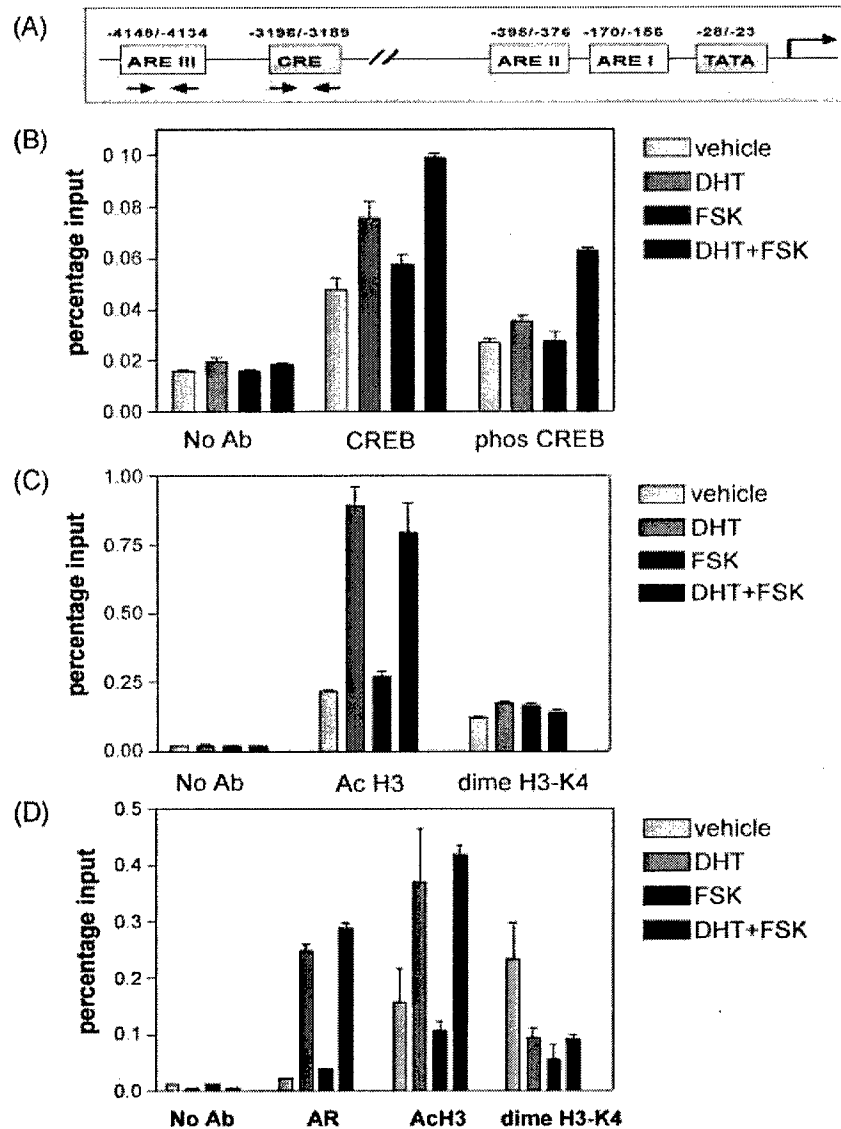
#### CREB binds to CRE at the PSA 5' regulatory region concomitant with histone acetylation

An additional rationale for CREB involvement in AR/PKA signaling at the PSA locus is provided by the fact that a putative CREB-binding site, CRE, is present



**Figure 3** PKA activation does not affect AR protein expression but significantly increases CREB phosphorylation when combined with AR signaling. (A) LNCaP cells were treated with either 10 nM DHT or 1  $\mu$ M forskolin (FSK), or both for 16 h and immunoblotted with anti-AR (N20) antibody. (B) For anti-CREB and antiphosphorylated CREB (pCREB) Western blot, LNCaP cells were treated with 10 nM DHT and/or 1  $\mu$ M or 50  $\mu$ M FSK for 10 min and immunoblotted with anti-CREB and anti-pCREB antibodies. Protein extracts in each case were loaded in equal amounts, as apparent by actin blotting. The number below each lane of protein expression indicates the relative intensity of each band normalized to corresponding actin expression level. The CREB and pCREB analyses are representative of four independent experiments.

at the 5' upstream regulatory region of the PSA gene (Sadar *et al.* 1999). Although no characterization of this specific CRE exists in the literature, it is known to be a perfect 8 bp palindromic site (TGACGTCA) located at -3196/-3189 of the PSA gene (Fig. 4A). PSA CRE is apart from the AR binding sites located at the enhancer (-4148/-4134) and promoter (-170/-156). To investigate the role of CREB in AR-mediated transcription of the PSA gene, we chromatin immunoprecipitated (ChIP) for CREB binding and histone modifications at PSA CRE. Remarkably, quantitative analysis of CREB and phosphorylated CREB occupancy revealed that the



**Figure 4** Phosphorylated CREB binds to CRE at the PSA 5' regulatory region, which is concomitantly histone acetylated. (A) Real-time PCR primers and probes were designed around PSA enhancer ARE site (-4148/-4134) and CRE (-3196/-3189) for chromatin immunoprecipitation (ChIP) assay. Chromatin was sonicated to around a 500 bp fragment. (B) CREB and phosphorylated CREB occupancies at the PSA CRE site after treatment with vehicle, 10 nM DHT and/or 1  $\mu$ M FSK. ChIP assay values are given as percentage of whole cell lysate (input). (C) Acetylation of histone H3 (Ac H3) and dimethylation of histone H3 at lysine 4 (dime H3-K4) at the CRE site measured by ChIP assay. The same conditions were used as above. (D) AR occupancy, acetylation of histone H3 and dimethylation of histone H3 at lysine 4 at the PSA enhancer ARE measured by ChIP assay. The same conditions were used as above. Values are means  $\pm$  S.E.M. of triplicate determinations and are representative of three independent experiments.

activated form of CREB was bound to the CRE only after DHT and forskolin exposure (Fig. 4B). Although the recruitment of activated CREB was low, the results were reproducible and in parallel with the low occupancy level of AR at the androgen response element

(ARE) sites located in the promoter (Jia *et al.* 2003, Louie *et al.* 2003). We also examined acetylation of histone H3 (Fig. 4C) and H4 (data not shown) at the PSA CRE region and found that both were acetylated by DHT stimulation, suggesting that transcriptional potential is

increased at the DNA element. Although the combination of DHT and forskolin treatment also yielded histone acetylation, the absence of any significant change compared with the DHT treatment alone, taken together with the absence of acetylation with the forskolin exposure alone, may imply that acetylation of histones is a DHT-specific effect. Since we did not observe phosphorylated CREB occupancy with DHT treatment, these results raised the possibility that the immunoprecipitated CRE chromatin fragment contained the nearby ARE of the enhancer (~1 kb upstream of CRE) due to inefficient sonication. However, comparison of the dimethylation pattern of histone H3 at lysine 4 at the enhancer ARE (Fig. 4D) with that at the CRE (Fig. 4C) demonstrated that the two immunoprecipitated chromatin fragments were distinct. This is consistent with our previous finding that dimethylation pattern dynamically varies with transcription activation at the PSA AREs, but not at other regions (Kim *et al.* 2003). In addition, AR occupancy was not detected at the CRE, and CREB binding was not apparent at the enhancer ARE (data not shown), a finding which further supported the improbability of immunoprecipitating the same chromatin fragment. Therefore, it was apparent that an interesting cross-talk between AR and PKA signaling occurred at the CRE.

#### CBP/p300 mediates interaction between AR and CREB

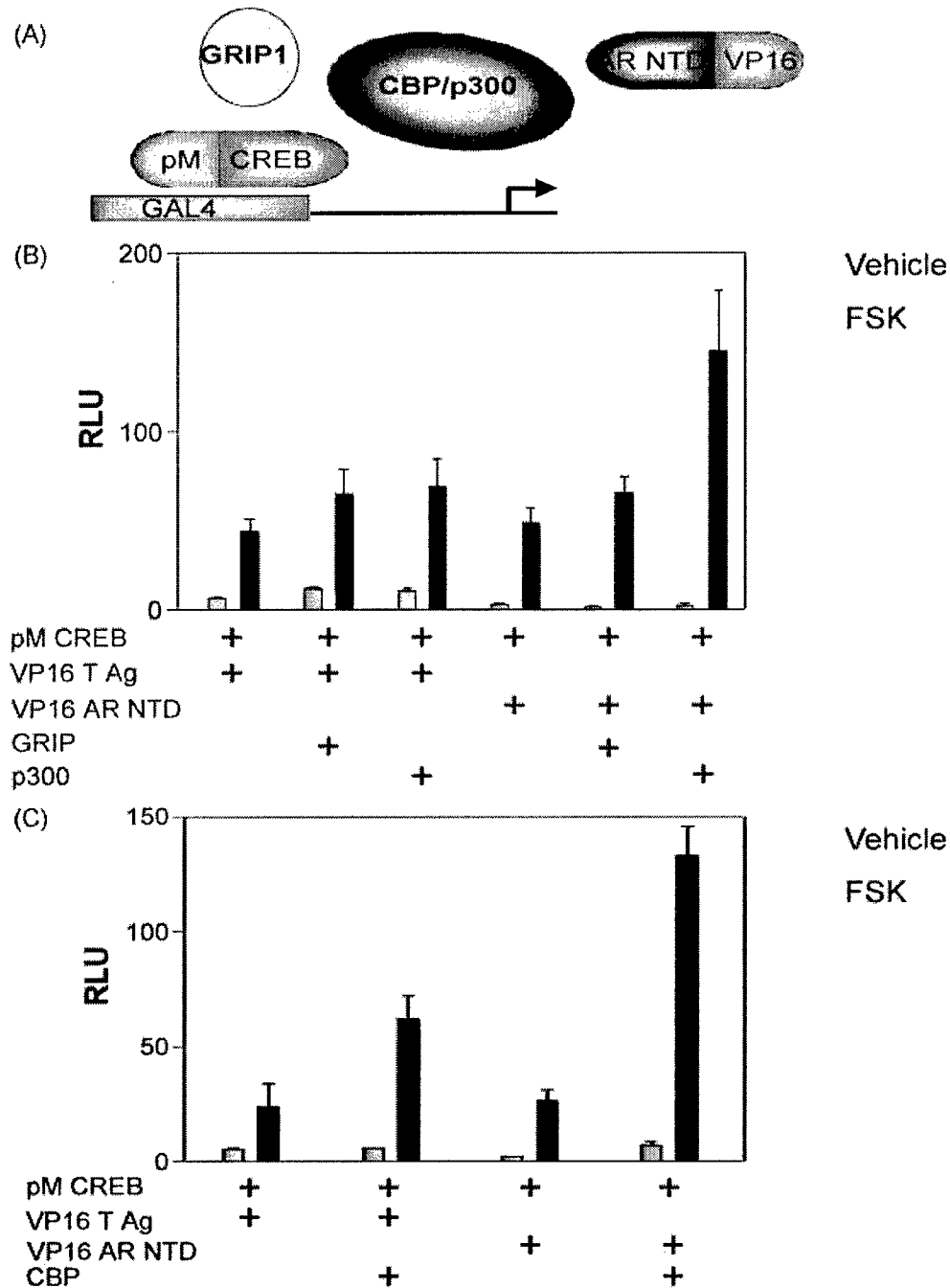
Subsequently, we investigated whether a physical interaction between AR and CREB was responsible for the observed cross-talk. To test a possible AR and CREB interaction, we utilized the mammalian two-hybrid system in Cos 7 cells, which lack an endogenously expressed AR. While both AR and CREB can bind to CBP/p300 (Chrivia *et al.* 1993, Lundblad *et al.* 1995, Aarnisalo *et al.* 1998, Debes *et al.* 2002), no evidence of direct interaction between AR and CREB exists. Thus, we also examined the possibility that CBP/p300 physically bridges the interaction between AR and CREB. Full-length CREB was cloned into the GAL4 DNA-binding domain plasmid (pM-CREB), and N-terminal domain of AR was cloned into VP16 activation domain plasmid (VP16-AR NTD) (Fig. 5A). When CREB and a control vector (VP16 T Ag) were cotransfected in a mammalian two-hybrid assay (Fig. 5B), intrinsic activation by the forskolin treatment was observed, probably due to the transcriptional activation domain present in the CREB protein. As this activity does not reflect an interaction between CREB and the control protein, we determined this level to be the background, with which other interactions would be compared. Cotransfection of coactivators GRIP1 or p300 did not indicate bridging of interaction between CREB and the control protein. When CREB was

cotransfected with AR NTD, no direct interaction was observed. Moreover, GRIP1 did not bridge the interaction between CREB and AR. However, cotransfection of p300 along with CREB and AR yielded a twofold increase in activity, suggesting that p300 can bridge the interaction between CREB and AR. This result is not due to p300's enhancing the transcriptional readout of GAL4 DNA-bound CREB, since p300 cotransfected with CREB and control protein did not produce a higher activity. Similar results were obtained when CBP was cotransfected (Fig. 5C), indicating that both CBP and p300 are able to bridge the interaction between CREB and AR. Notably, when CREB and ligand-binding domain of AR (AR-LBD) were coexpressed, CBP and p300 could not bridge the interaction (data not shown). This is consistent with the previously reported finding that CBP/p300 interacts with only the N-terminal domain of AR (Fronsdal *et al.* 1998). Therefore, the data presented suggest a possible indirect AR/CREB interaction mediated by CBP/p300.

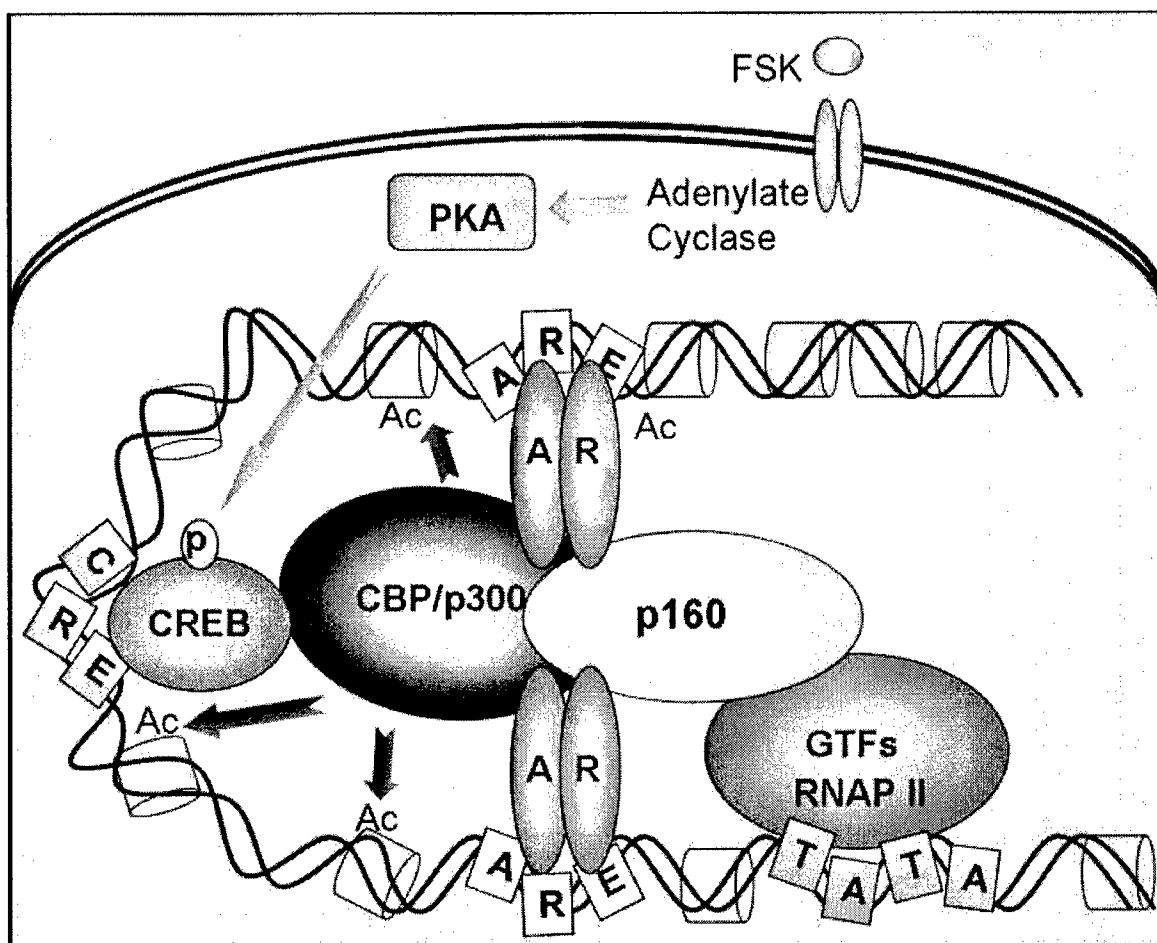
#### Discussion

The AR plays a central role in the biology of prostate cancer, and androgen ablation therapy remains, after more than 50 years, the most effective treatment for advanced prostate cancer. However, prostate tumors treated with androgen ablation invariably progress to the therapy-resistant form that cannot be further treated with success. Although the tumors are androgen-independent at this final stage, a functional AR signaling pathway is maintained. Various molecular bases for the progression from androgen-dependent prostate cancer to the advanced disease have been proposed. Here, we illustrated a novel mechanism of cross-talk between AR and PKA signaling pathways, which cooperate to activate androgen-responsive gene transcription. Our data confirmed that PKA activation enhances androgen-dependent transcription from both transiently transfected and chromatin-integrated genes. A proposed consequence of PKA activation is thought to be the phosphorylation of AR, which in turn could render the protein more transcriptionally active. However, in addition to the conflicting result, indicating that PKA activation may actually dephosphorylate AR (Blok *et al.* 1998), a recent study showed that mutation of the phosphorylation sites does not have an effect on AR transcriptional activity (Gioeli *et al.* 2002). Moreover, the *in vivo* phosphorylation sites may regulate nuclear transport, and not transcription (Black *et al.* 2004). Our evidence suggests that PKA enhancement of androgen-mediated transcription is not due to increased AR expression or stabilization.

Contrary to the finding by Sadar *et al.* (1999), forskolin did not stimulate androgen-independent transcription in



**Figure 5** CBP/p300 mediates interaction between AR and CREB. (A) Mammalian two-hybrid assay scheme. Full-length CREB was inserted into GAL4 DNA binding pM plasmid, and N-terminal domain of AR (AR-NTD, aa 1–538) was cloned into VP16 activation domain plasmid. Luciferase reporter driven by GAL4 (PGK1), pM-CREB, VP16-AR NTD, GRIP1, CBP and/or p300 plasmids was cotransfected into Cos 7 cells lacking an endogenous AR. (B) p300, but not GRIP1, mediates the interaction between AR and CREB. Cos 7 cells were transfected with pM-CREB, VP16 T Ag (control) or VP16-AR NTD, GRIP1, and/or p300. Cos 7 cells were treated with vehicle or 10  $\mu$ M forskolin (FSK). The values are represented as relative light units (RLU). (C) CBP mediates the interaction between AR and CREB. The same conditions as above were used in the experiment with CBP. Values are means  $\pm$  S.E.M. of quadruplicate determinations, and the data are representative of three experiments.



**Figure 6** Proposed model of cross-talk between AR and PKA signaling pathway at the 5' transcriptional control regions of the PSA gene. In this model based on the experiments illustrated in this study, PKA signaling phosphorylates and activates CREB, which binds to the PSA CRE site after DHT-induced AR signaling recruits CBP/p300, which mediates the interaction between AR and CREB. The recruitment of CBP/p300 acetylates the histones around the AREs and CRE. This higher-ordered enhanceosome assembly may stabilize the interaction with general transcription factors (GTFs) and RNA polymerase II (RNAP II), and thus increase the transcriptional potential at the PSA promoter.

our experiments. A possible explanation is that we tested early-passage (passages 19–24) LNCaP cells while the Sadar group used later-passage cells (passages 44–55). Taken together with the observation that CBP/p300 overexpression is sufficient to induce PKA-mediated transcription that is independent of DHT, our results suggest the interesting possibility that LNCaP cells may progressively express more CBP/p300 over time to compensate for the low-androgen environment. Activation of PKA signaling and increased expression of p300/CBP may be sufficient for progression of LNCaP cells toward androgen independence with respect to growth, gene expression and tumorigenicity. CBP and p300 are transcriptional coactivators that are fundamentally important in various signal-regulated transcription events. CBP was first identified as a protein that interacts with CREB, while p300 was isolated as a crucial cellular

protein targeted by the adenovirus E1A oncoprotein (Whyte *et al.* 1989, Chrivia *et al.* 1993). Yet, CBP and p300 are highly related in structure and interact with a similar set of proteins, including p160 coactivators, general transcriptional apparatus, and nuclear hormone receptors (Giordano & Avantaggiati 1999). Since CBP/p300 does not bind to DNA elements directly, we examined whether another transcription factor was mediating the PKA effect. An attractive candidate was the CREB protein because of its association with CBP/p300 and its activation via the PKA pathway. Western blot data indicated that CREB phosphorylation is significantly increased by DHT and forskolin, corresponding to the observed enhancement of PSA transcription.

Moreover, ChIP assays demonstrated a novel occupancy of CREB at the PSA CRE site, which was

accompanied by histone acetylation. Interestingly, only the combination of DHT and forskolin treatment recruited activated CREB to CRE, further supporting a cross-talk between AR and PKA signaling. This phenomenon may reflect the significantly increased level of phosphorylated CREB induced by DHT and forskolin treatment of LNCaP cells, as evidenced by the Western blot data. Although the  $K_d$  for CREB binding to palindromic CREs is low (1 nM) compared with the nuclear concentration of the protein (100 nM), the CRE is generally less active when moved further upstream of the promoter (Shaywitz & Greenberg 1999). Thus, the increased availability of phosphorylated CREB may be a crucial event mediated by AR and PKA cross-talk. In addition, the mammalian two-hybrid data that suggest CBP/p300 is able to mediate the interaction between AR and CREB, taken together with our ChIP results, may indicate that AR and CREB form an enhanceosome at the 5' regulatory regions of the PSA gene (Fig. 6). In this model, stimulation of androgen and the PKA pathway activates both AR and CREB, which recruit CBP/p300, forming a more stable tertiary interaction in an enhanceosome. As the specific recruitment of a complex with HAT activity to a promoter may play a critical role in overcoming the repressive effects of chromatin structure on transcription (Struhl *et al.* 1998), a higher-ordered assembly that recruits histone acetylation to the regulatory elements could increase the transcription potential. CREB may also function cooperatively with CBP/p300 to regulate AR activity, in part by enhancing the recruitment of TAF<sub>II</sub> 130 to the promoter (Nakajima *et al.* 1997, Felinski & Quinn 1999).

As androgen and the AR play important roles in prostate cancer progression, understanding the factors involved in the regulation of androgen/AR action may provide molecular targets for prostate cancer treatment. Aberrant activation of the AR through the PKA signaling pathway may be responsible for the progression of prostate tumors to the rapidly proliferating, androgen-independent state. Further investigation of the PKA signaling effect on AR activity may yield valuable information potentially leading to viable therapies in the future.

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# Androgen Receptor–Dependent *PSA* Expression in Androgen-Independent Prostate Cancer Cells Does Not Involve Androgen Receptor Occupancy of the *PSA* Locus

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## Abstract

It is widely suspected that androgen-independent prostate cancer growth depends on androgen receptor signaling via ill-defined mechanisms. Prostate-specific antigen (*PSA*) expression is often used to measure androgen receptor activity in cells and prostate cancer progression in patients. In the present study, we have compared androgen receptor activity using *PSA* and human male germ cell-associated kinase (*hMAK*), as read-outs in androgen-dependent LNCaP and androgen-independent C4-2B cells. As expected, very little *PSA* and *hMAK* expression were detected in LNCaP cells in the absence of androgens, whereas substantial expression of *PSA* was observed only in C4-2B cells under the same conditions. The addition of dihydrotestosterone to the culture medium increased the expression of both genes in both cell types. Comprehensive chromatin immunoprecipitation analysis of the entire *PSA* locus and an androgen-response element in *hMAK* unexpectedly revealed that androgen receptor was not occupying any site in the absence of dihydrotestosterone in either cell type. In line with the expression data, and in the absence of dihydrotestosterone, histone acetylation and RNA polymerase II occupancy was substantial at the *PSA* locus in C4-2B but not in LNCaP cells. In the presence of dihydrotestosterone, androgen receptor was found to occupy mainly the enhancer region of *PSA* in both cell types, accompanied with increases in histone acetylation and RNA polymerase II occupancy. Although the androgen receptor was not directly involved in the androgen-independent expression of *PSA* in C4-2B cells, small interfering RNA knock-down of androgen receptor significantly reduced *PSA* expression in both the presence and absence of dihydrotestosterone. In contrast, *hMAK* expression was decreased only in the presence of dihydrotestosterone after androgen receptor knock-down. We conclude that androgen-independent expression of *PSA* in C4-2B cells does not rely on the direct occupancy of the androgen receptor at the *PSA* locus, but is nevertheless affected indirectly via unknown androgen receptor–dependent mechanism(s) that influence the expression from some but not all androgen receptor target genes. (Cancer Res 2005; 65(17): 1-7)

## Introduction

Androgen ablation therapy of prostate cancer, although initially efficacious, nearly always leads to treatment resistance and

progression of the disease (1). Although the disease is then referred to as androgen-independent, it simply reflects resistance to the original therapy protocol. Mechanisms underlying androgen independence are thought to involve an adaptation of androgen receptor signaling to function under low or absent androgen levels (2). Prostate-specific antigen (*PSA*) is an androgen-responsive gene and its expression is often used as a measure of androgen receptor signaling and as a monitor of prostate cancer progression (3). Human male germ cell-associated kinase (*hMAK*) is a newly identified target gene of the androgen receptor that may participate in prostate cancer cell physiology (4). Because the role of the androgen receptor in androgen-independent prostate cancer is not clearly defined, we have embarked on a detailed analysis of androgen receptor signaling measuring *PSA* and *hMAK* expression in LNCaP and C4-2B cells. LNCaP cells were originally obtained from a needle aspiration biopsy of the left supraclavicular lymph node of a 50-year-old Caucasian male with confirmed diagnosis of metastatic prostate cancer (5). The cells are responsive to dihydrotestosterone in terms of growth and *PSA* expression. Although the androgen receptor in LNCaP cells is mutated (6), it is generally normal with respect to transactivation activities (7). C4-2B is a subline of LNCaP obtained by passage and growth in castrated athymic mice (8). The cells are no longer responsive to hormone manipulation in animals (although they still express a functional androgen receptor; ref. 9) and metastasize readily to lymph nodes and bone in athymic mice. Our results reveal the striking finding that androgen receptor–dependent *PSA* expression in C4-2B cells does not involve androgen receptor occupancy at the *PSA* locus.

## Materials and Methods

**Cell culture and materials.** Human prostate cancer LNCaP cells, obtained from the American Type Culture Collection (Manassas, VA) and C4-2B, obtained from ViroMed Laboratories (Minneapolis, MN), were both maintained in RPMI 1640 supplemented with 5% (v/v) fetal bovine serum. 5 $\alpha$ -Dihydrotestosterone was purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies were anti-androgen receptor NH<sub>2</sub>-terminal (N20), anti-androgen receptor COOH-terminal (C19) anti-Pol II (N20), anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Ach3 (Upstate Biotechnology, Inc., Lake Placid, NY) and anti-*PSA* (DAKO Corp., Carpinteria, CA). Immunoblotting was done as described previously (10), with the indicated antibodies.

**Real-time reverse transcription-PCR.** After the indicated treatments of LNCaP and C4-2B cells, total cellular RNA was prepared using SV Total RNA Isolation System (Promega, Madison, WI). A two-step reverse transcription-PCR method was employed using the TaqMan Gold RT-PCR kit (Applied Biosystems, Branchburg, NJ) and primers and probes for *PSA* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression were as previously described (10). Primers and probe for *hMAK* expression were: 5'-GGTCACAGCCACCATACTGA-3' (forward); 5'-ACTTCCAACAGCC-CACA-CATC-3' (reverse); 5'-6-FAM-CCAGATGGTATCGTGCCCTGAAGT-TT-BHQ-1-3' (probe). Triplicate PCR reactions were done. *GAPDH* mRNA

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expression was analyzed for each sample in parallel. The results are represented as *PSA* or *hMAK/GAPDH* mRNA ratios.

**Transient transfection and luciferase assays.** LNCaP ( $6 \times 10^5$  cells/well) and C4-2B ( $3 \times 10^5$  cells/well) were plated in six-well plates and grown in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped fetal bovine serum (CSS) for 2 days. Cells were then transfected with an androgen receptor-responsive reporter, pGL3-PSA5.85 (2  $\mu$ g/well) using LipofectAMINE 2000 (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's protocol. PGL3-PSA5.85 (a gift from Dr. Hong-Wu Cheng) was constructed by inserting the entire 5.85 kb PSA upstream sequence into pGL3 basic vector (11). After transfection, cells were grown in phenol red-free RPMI 1640 containing 5% CSS with dihydrotestosterone or vehicle control for 24 hours. Luciferase activity determinations in cell lysates were conducted as previously described (10).

**Chromatin immunoprecipitation assays.** LNCaP ( $6 \times 10^6$  cells/150 mm dish) and C4-2B ( $3 \times 10^6$  cells/150 mm dish) were cultured in phenol red-free RPMI 1640 supplemented with 5% CSS for 3 days. Cells were treated with dihydrotestosterone or vehicle control for 4 hours, and chromatin immunoprecipitation (ChIP) assays were conducted as described previously (10). DNA samples from ChIP preparations were analyzed by real-time PCR using an iCycler optical system (Bio-Rad, Hercules, CA) and AmpliTaq Gold PCR master mix (Applied Biosystems). The primers and probes (synthesized by Biosearch Technologies, Novata, CA) were (as they relate to the positions at the *PSA* locus; see Fig. 2):

- A 5'-GCCTGGATCTGAGAGAGATATCATC-3' (forward)  
5'-ACACCTTT-TTTTTCTGGATTGTG-3' (reverse)  
5'-6-FAM-TGCAAGGATGCCTGCTTTACAAACATCC-BHQ-1-3' (probe)
- B 5'-CTGGGCAAGCACAATCTGA-3' (forward)  
5'-GTGTGATGCTGGATGATGAGTG-3' (reverse)  
5'-6-FAM-CATGAAAGATGCCCCAGAGGCCTTG-BHQ-1-3' (probe)
- C 5'-CAGTGGCCATGAGTTTGTGTTG-3' (forward)  
5'-AACCAATCCAACCTGCATTATACACA-3' (reverse)  
5'-6-FAM-CCCAACG-CAACTTAACCTAACAAG-BHQ-1-3' (probe)
- D 5'-TTCCAGGCTGGAGTTCAATAG-3' (forward)  
5'-GGAGACCGAAGCAGGAGAATC-3' (reverse)  
5'-6-FAM-CAGCTCACTGTAGCCTTGAACCTCTGGC-BHQ-1-3' (probe)
- E 5'-CCTAGATGAAGTCTCCATGAGCTACA-3' (forward)  
5'-GGGAGGGAGA-GCTAGCACTTG-3' (reverse)  
5'-6-FAM-CAATTACTAGATCACCCTGGATGCACCAGG-BHQ-1-3' (probe)
- F 5'-CACACCCGCTCTACGATATGAG-3' (forward)  
5'-GAGCTCGGCAGGCTCTGA-3' (reverse)  
5'-6-FAM-CTCAGCCACGACCTCATGCTGCT-BHQ-1-3' (probe)
- G 5'-TCATCATGAATCGCACTGTTAGC-3' (forward)  
5'-GCCCAAGTGCCTTGGTATACC-3' (reverse)  
5'-6-FAM-TGAATCATCTGGCAGGCCCAA-BHQ-1-3' (probe)

The primers and probes (synthesized by Biosearch Technologies) were (as they relate to the indicated *hMAK* locus; see Fig. 4):

- 5'-AGAAGTTGGAGACGACCCTGAA-3' (forward)
- 5'-CAACTAGAGAATCAACGATGGAGTCA-3' (reverse)
- 5'-6-FAM-CCAACAACATCAAGCCAAATGCAACTATTG-BHQ-1-3' (probe).

Duplicate immunoprecipitations for each antibody and triplicate PCR reactions for each immunoprecipitation sample were done. The results are given as a percentage of input.

**Small interfering RNA transfection.** C4-2B cells ( $1.5 \times 10^5$  cells/well) were plated in six-well plates and grown in phenol red-free RPMI 1640 containing 5% CSS for 2 days. Cells were transfected with one of two

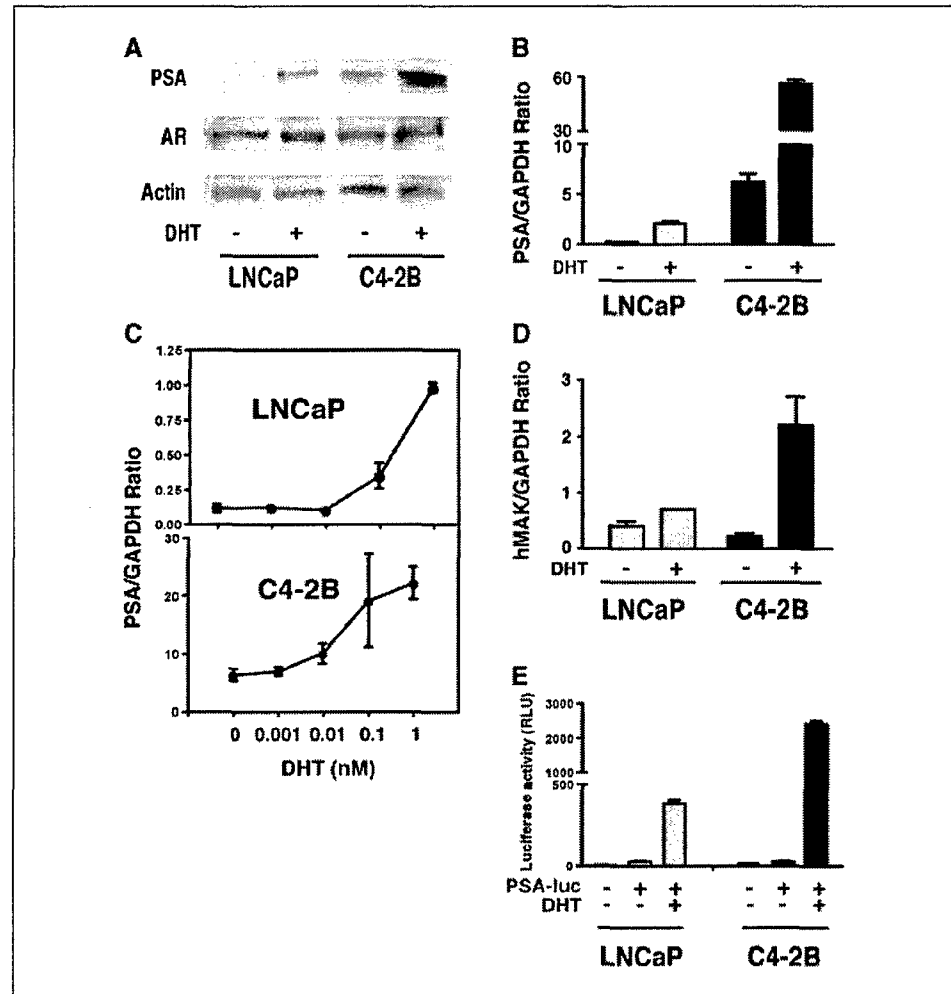
androgen receptor small interfering RNA (siRNA) duplexes. The first was sense, 5'-AUG UCA ACU CCA GGA UGC UTT-3'; and antisense, 5'-AGC AUC CUG GAG UUG ACA UTT-3', directed against the coding region of androgen receptor mRNA. The second was sense, 5'-ACG UUU ACU UAU CUU AUG CTT-3'; and antisense, 5'-GCA UAA GAU AAG UAA ACG UTT-3', directed against the 3'-untranslated region of androgen receptor mRNA. A nonspecific siRNA duplex, 5'-AGA UCU GGC UAU CGC GGU ATT-3' and 5'-UAC CGC GAU AGC CAG AUC UTT-3' was used as a control. All siRNA duplexes were at a final concentration of 100 nmol/L and transfected using OligofectAMINE reagent (Invitrogen) according to the manufacturer's instructions. After transfection, cells were grown in phenol red-free RPMI 1640 containing 5% CSS for 48 hours and then treated with dihydrotestosterone or vehicle control for 18 hours. Total RNA and protein extractions were conducted for reverse transcription-PCR and immunoblot analyses, respectively, as described above.

## Results

**PSA and hMAK expression in LNCaP and C4-2B cells.** In LNCaP cells, virtually no detectable PSA expression was observed in the absence of androgens as measured by immunoblot of the PSA protein (Fig. 1A) or expression of PSA mRNA (Fig. 1B and C). On the other hand, PSA expression was substantial in C4-2B cells in the absence of androgens (Fig. 1A-C). In contrast, the low level of *hMAK* expression in the absence of dihydrotestosterone stimulation was not higher in C4-2B cells compared with LNCaP (Fig. 1D). Addition of dihydrotestosterone increased the expression of *PSA* and *hMAK* in both cell lines; expression values of *PSA* in C4-2B cells in the absence of dihydrotestosterone were similar or even higher than values in LNCaP cells in the presence of androgens. Even at low concentrations of dihydrotestosterone, C4-2B expressed more than an order of magnitude higher *PSA* mRNA than did LNCaP cells at any given dihydrotestosterone concentration (Fig. 1C). However, when a transiently-transfected PSA-driven luciferase reporter was used, virtually no detectable luciferase expression was measured in the absence of androgens in either LNCaP cells or C4-2B cells (Fig. 1E). Therefore, the observed ligand-independent expression of endogenous PSA in C4-2B cells is somehow related to the chromatin-integrated nature of the native *PSA* gene (see below).

**Chromatin immunoprecipitation analysis of the PSA and hMAK loci.** In previous work, we began to define transcriptional activity at the *PSA* locus in terms of androgen receptor and cofactor occupancies and histone modifications (7, 10, 12). We have now extended the analysis to interrogate seven sites at the locus spanning the enhancer, promoter, an exon and 3'-untranslated region of the gene (Fig. 2A). Androgen receptor recruitment, histone H3 acetylation and RNA polymerase II occupancy were analyzed in LNCaP and C4-2B cells in the absence or presence of dihydrotestosterone stimulation (Fig. 2B). The IgG-negative controls gave very low signals throughout in both cell lines. No androgen receptor occupancy was detected in either cell line in the absence of dihydrotestosterone using an NH<sub>2</sub>-terminal androgen receptor antibody (N20; Fig. 2B) or a COOH-terminal antibody (C19; Fig. 3), whereas significant occupancy was observed at the enhancer (A) and to a lesser extent at the promoter (E) in both cell lines in the presence of dihydrotestosterone (Figs. 2B and 3). This is an interesting finding considering the high expression levels of PSA in C4-2B cells in the absence and presence of dihydrotestosterone (orders of magnitude higher than in LNCaP cells), yet no androgen receptor was found at the *PSA* locus in the absence of dihydrotestosterone, and in the presence of dihydrotestosterone,

**Figure 1.** PSA expression in LNCaP cells and C4-2B cells. **A**, LNCaP and C4-2B cells were incubated in phenol red-free RPMI 1640 containing 5% CSS for 3 days and then treated with dihydrotestosterone (10 nmol/L) or vehicle control (ethanol) for 18 hours. Immunoblots were conducted using anti-PSA, anti-androgen receptor and anti-actin antibodies. **B**, LNCaP cells and C4-2B cells were treated as in (A). PSA and GAPDH mRNA levels were measured by real-time reverse transcription-PCR. The PSA expression values are shown as PSA/GAPDH mRNA ratios. Values are presented as means  $\pm$  SD of triplicate real-time PCR assays. **C**, LNCaP and C4-2B cells were incubated in phenol red-free RPMI 1640 containing 5% CSS for 3 days and then treated with different concentrations of dihydrotestosterone as indicated for 18 hours. PSA and GAPDH mRNA levels were measured as in (B). **D**, cDNA obtained from (B), was analyzed for *hMAK* mRNA levels. **E**, LNCaP and C4-2B cells were transiently transfected with pGL3-PSA5.85 and incubated with dihydrotestosterone (10 nmol/L) or vehicle control for 24 hours. Values are presented as the means  $\pm$  SD of duplicate wells. Triplicate samples of each well was read in a luminometer, and the average was normalized to total protein concentration. RLU, relative luciferase units.



androgen receptor occupancy was lower in C4-2B than in LNCaP cells. The fact that this result was obtained using androgen receptor antibodies directed to two different epitopes indicated that it is unlikely that epitope masking is the reason for the lack of androgen receptor detection at these loci in the absence of dihydrotestosterone treatment. The results, therefore, indicate that a significant proportion of PSA expression occurs either via non-androgen receptor mechanisms or androgen receptor-dependent mechanisms not related to androgen receptor occupancy at the *PSA* locus in C4-2B cells. Histone H3 acetylation and RNA polymerase II occupancy was substantially higher in C4-2B than LNCaP cells in the absence of dihydrotestosterone (at A, B, E, and F sites) in line with dihydrotestosterone-independent expression of PSA in these cells. This indicates that these regions, especially E and F, are in readily accessible chromatin, which likely facilitate the recruitment of general basal transcription factors and RNA polymerase II to mediate the observed transcription of PSA mRNA. Dihydrotestosterone addition resulted in a further increase of these variables in C4-2B cells, which was now also detected in LNCaP cells. Overall, these results indicate that PSA expression in the absence of dihydrotestosterone occurs in C4-2B cells in the absence of androgen receptor occupancy at this locus.

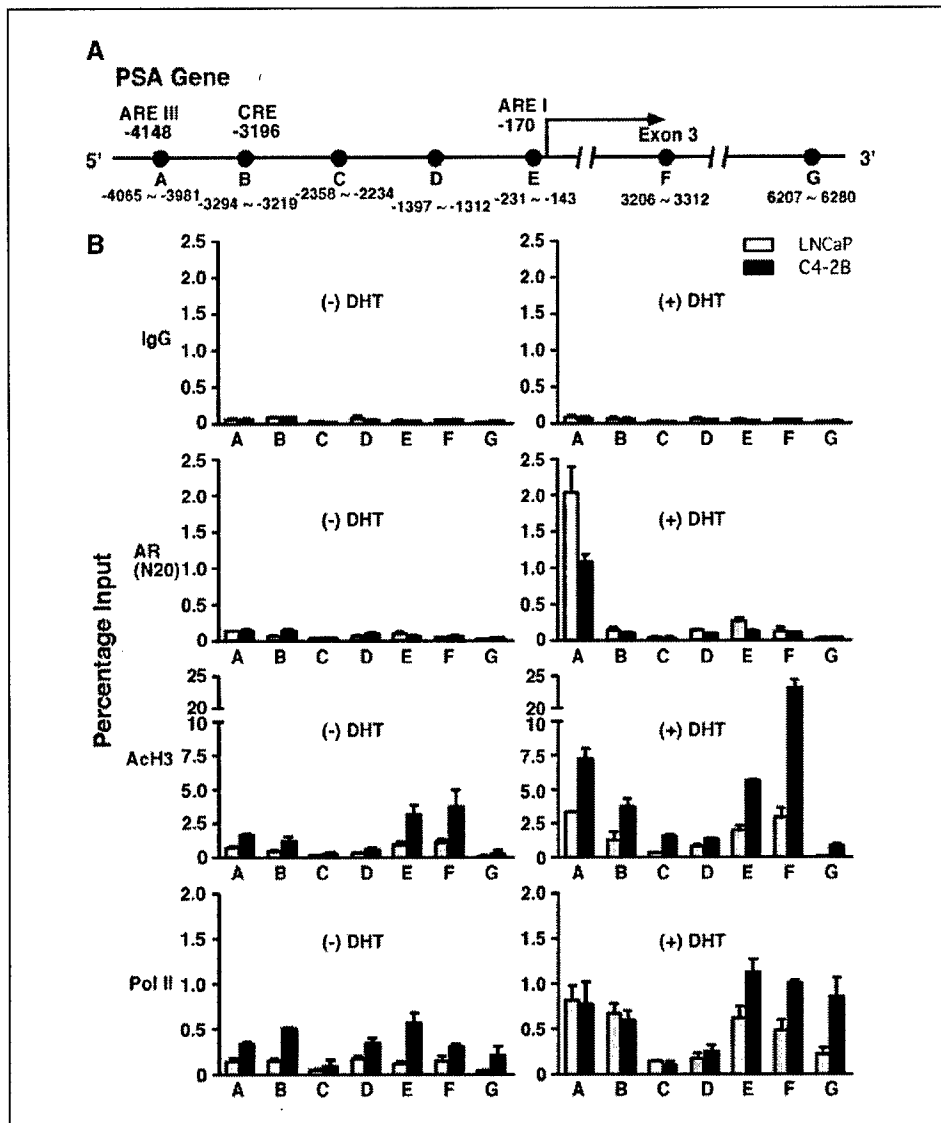
The same ChIP DNA obtained for the experiment depicted in Fig. 2 was assessed for an androgen-response element located

about 3.5 kb upstream from the transcription start site of the *hMAK* gene (Fig. 4).

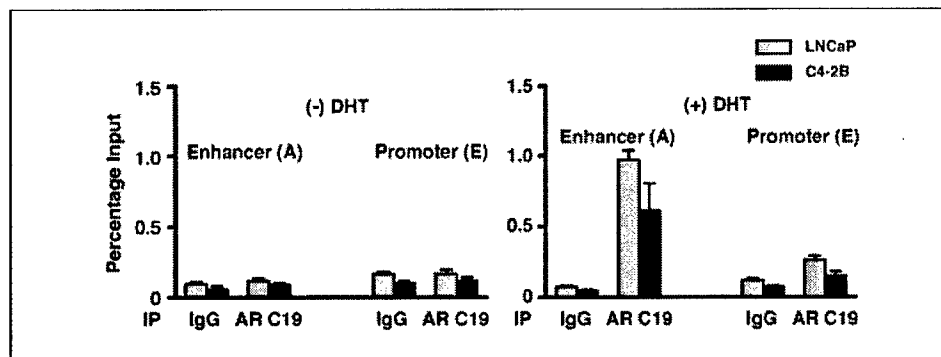
The androgen-response element (GGAACATGATGGCCT) was identified by scanning the 5'-upstream region of the *hMAK* gene using ConSite (web-based prediction of regulatory elements; ref. 13). In the absence of dihydrotestosterone, very low to negligible androgen receptor occupancy, histone acetylation or polymerase II occupancy were detected at this site in both cell lines consistent with the very low expression levels of this gene under these conditions. Note that this result is in contrast with the observations at the *PSA* locus in C4-2B cells. The addition of dihydrotestosterone to the medium resulted in increases in all the parameters, especially in C4-2B cells, again consistent with expression values obtained for this gene under these conditions. The only inconsistency noted between ChIP results and expression levels of the two genes is the lack of androgen receptor occupancy at the *PSA* enhancer in the absence of dihydrotestosterone in C4-2B cells, whereas substantial expression of *PSA* occurred under these conditions in the cells. We therefore tested whether the androgen receptor is even involved under these conditions.

**Androgen receptor involvement in *PSA* and *hMAK* expression levels in the absence of dihydrotestosterone in C4-2B cells.** To determine whether the androgen receptor was involved, even indirectly, in PSA expression in the absence of dihydrotestosterone

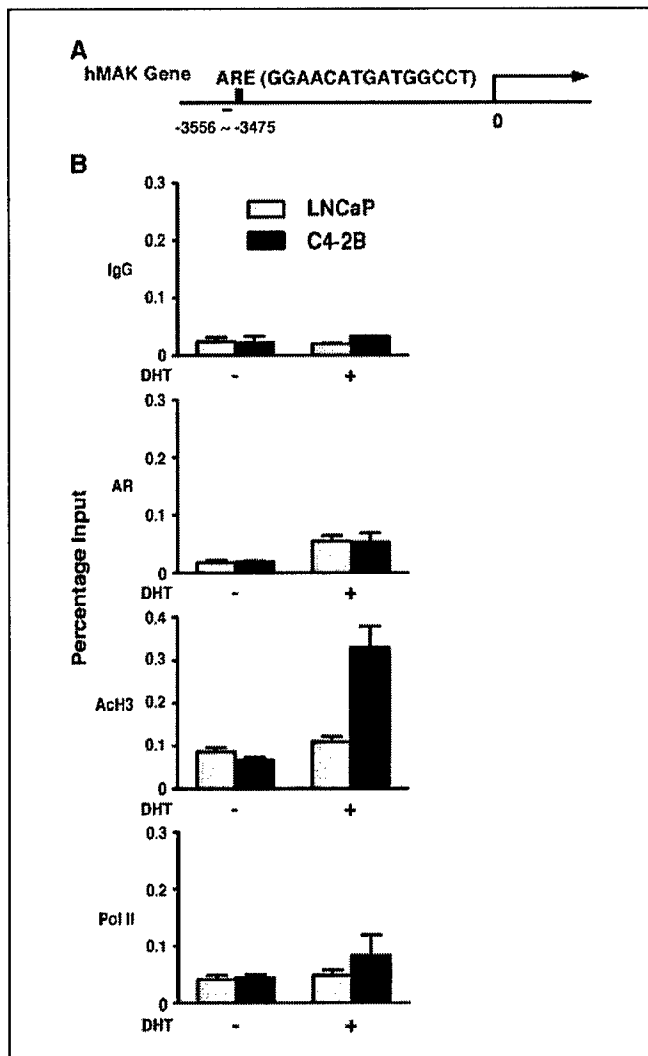
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**Figure 2.** ChIP analysis at *PSA* locus. **A**, schematic representation of the *PSA* gene. ARE, androgen response element; CRE, cyclic AMP-responsive element; ●, real-time PCR targeted regions; arrow, transcription start site. **B**, LNCaP and C4-2B cells were incubated in phenol red-free RPMI 1640 containing 5% CSS for 3 days and then treated with or without dihydrotestosterone (10 nmol/L) for 4 hours. Androgen receptor occupancy (using N20 antibody), histone H3 acetylation and RNA Pol II recruitment were examined by ChIP analyses. The value of each immunoprecipitation sample was obtained from the average of triplicate real-time PCR assays from duplicate immunoprecipitations; columns, mean; bars,  $\pm$  SD. Input values were obtained from samples treated in the same way as the experimental ones, except that no immunoprecipitation steps were done.

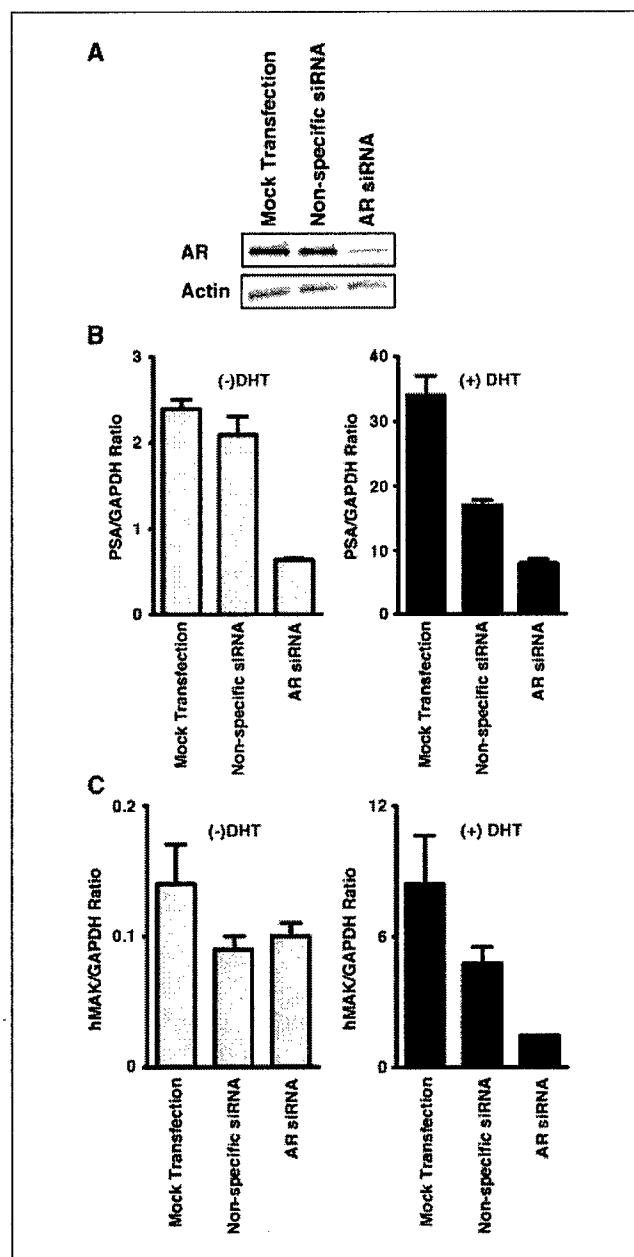


**Figure 3.** Androgen receptor ChIP with COOH-terminal androgen receptor antibody. LNCaP and C4-2B cells were incubated in phenol red-free RPMI 1640 containing 5% CSS for 3 days and then treated with or without dihydrotestosterone (10 nmol/L) for 4 hours. Androgen receptor occupancies on *PSA* enhancer and promoter were examined by ChIP analyses using the antibody against androgen receptor COOH terminus. The value of each immunoprecipitation sample was obtained from the average of triplicate real-time PCR assays from duplicate immunoprecipitations, and are presented as percentage input and represent mean values  $\pm$  SD. Input values were obtained from samples treated in the same way as the experimental ones, except that no immunoprecipitation steps were done.



**Figure 4.** ChIP analysis at *hMAK* locus. **A**, schematic representation of the *hMAK* gene. ARE, androgen response element; arrow, transcription start site. **B**, ChIP DNA samples obtained from the experiment depicted in Fig. 2B were analyzed for androgen receptor occupancy, histone H3 acetylation and RNA Pol II recruitment at the *hMAK* locus. The value of each immunoprecipitation sample was obtained from the average of triplicate real-time PCR assays from duplicate immunoprecipitations, and values are presented as percentage input and represent mean values  $\pm$  SD. Input values were obtained from samples treated in the same way as the experimental ones, except that no immunoprecipitation steps were done.

of the siRNA. Using the same cDNA, no equivalent decrease in the low but measurable level of *hMAK* expression was observed (Fig. 5C). The decrease in dihydrotestosterone-stimulated activity observed with the nonspecific siRNA treatment might be related to



**Figure 5.** PSA and *hMAK* expression in C4-2B cells after siRNA-mediated knock-down of androgen receptor. **A**, C4-2B cells were incubated in phenol red-free RPMI 1640 containing 5% CSS for 2 days and then mock-transfected or transfected with androgen receptor siRNA (100 nmol/L) directed against the coding region of the androgen receptor mRNA or the same amount of nonspecific siRNA. After 48 hours, androgen receptor protein levels were measured by immunoblot analysis. The membrane was stripped and reprobed with an anti-actin antibody. **B** and **C**, 48 hours after androgen receptor siRNA or nonspecific siRNA transfection, C4-2B cells were treated with or without dihydrotestosterone (10 nmol/L) for 18 hours. PSA (**B**) and *hMAK* (**C**) mRNA levels were determined by real-time reverse transcription-PCR. The PSA expression values are shown as PSA or *hMAK*/GAPDH mRNA ratios. Columns, mean; bars,  $\pm$  SD. Results shown are representative of three independent experiments. Similar results were obtained when a siRNA directed against the androgen receptor mRNA 3'-untranslated region was used.

**F5** in C4-2B cells, we knocked down the androgen receptor using a siRNA approach and measured PSA mRNA expression (Fig. 5). As a negative control, we also assayed the expression of *hMAK* using the same cDNA preparation, and as a positive control, the expression of these genes when the cells were stimulated by dihydrotestosterone. In the experiment depicted, we used the siRNA directed against the coding region of androgen receptor mRNA (see Materials and Methods). Androgen receptor was knocked-down successfully as revealed by immunoblot analysis (Fig. 5A). This maneuver caused a substantial inhibition of both androgen-dependent and -independent PSA expression roughly to the same extent as the androgen receptor protein was knocked down (Fig. 5B). Similar results were obtained using a siRNA directed against the androgen receptor mRNA 3'-untranslated region (data not shown), indicating that the results were not due to off-target effects

a general siRNA effect or possibly due to stimulation of the IFN system elicited by double-stranded RNA (14). This was not seen in the PSA expression activity in the absence of dihydrotestosterone. The fact that androgen receptor knock-down caused PSA expression inhibition (but not *hMAK* expression inhibition) in the absence of dihydrotestosterone, indicates that the androgen receptor is somehow involved in PSA expression specifically. The involvement, however, seems to be indirect because no androgen receptor occupancy at the androgen receptor enhancer was apparent in our ChIP analyses in the absence of dihydrotestosterone treatment in C4-2B cells (Figs. 2 and 3).

## Discussion

The androgen receptor signaling axis is involved in all phases of prostate cancer, from genetic predisposition through disease progression, including the development of resistance to androgen ablation therapies. Resistance to androgen ablation is not due to a loss of androgen responsiveness, but in most cases, is associated with altered androgen receptor activity (reviewed in refs. 2, 15, 16). Such aberrant androgen receptor signaling is thought to include androgen receptor hypersensitivity to androgens, androgen receptor insensitivity to antagonists, increased androgen receptor expression levels, or androgen receptor activity modulation by nonsteroidal signaling pathways. Although the details of these molecular processes are largely unknown, they may be linked, operating together in a given tumor. It was recently shown that increased androgen receptor expression levels are necessary and sufficient to convert prostate cancer to an ablation-resistant state (17).

The main finding of the present work, which adds another dimension to the notions referred to above, is that the androgen receptor is involved in PSA expression in the absence of androgen in the androgen-independent cell line, C4-2B, without occupying regulatory sites in the PSA upstream control region. What are the possible mechanisms to explain this? This question is not trivial because as stated above, androgen-independent receptor activity may be one of the main mechanisms governing growth in androgen-independent, advanced prostate cancer.

One possibility to explain our findings is that the androgen receptor may affect the transcription of other transcription factors that in turn activate PSA expression. Candidates for such indirect effects are general transcription factors like SP1 or transcription factors affected by non-steroid hormone signals like cyclic AMP-responsive element binding protein, for which

response elements exist at the PSA locus. We recently analyzed the involvement of cyclic AMP-responsive element binding protein at the PSA locus, and although we could define an enhanceosome with cooperation between the androgen receptor and cyclic AMP-responsive element binding protein via p300/CBP, its contribution to overall transcription was relatively small and could only be observed by protein kinase A stimulation with forskolin (18). This does not exclude the involvement of other transcriptional factors in the androgen-independent activation of PSA expression with which the androgen receptor may cooperate in an indirect fashion.

Another and even more intriguing mechanism of androgen receptor-dependent, but androgen-independent PSA expression, may be related to the nongenomic actions of the androgen receptor as proposed to be active in many mammalian cell types (19). Such nonclassical androgen receptor mechanisms may work via classic nonsteroidal signaling pathways affecting any number of other transcription factors. For example, it was reported that androgen receptor forms a triple complex containing androgen receptor/p85 $\alpha$ /Src affecting phosphoinositide-3-kinase/Akt activation and thus mediating androgen-induced cell growth and survival (20). More recently, it was reported that the Src-MEK-1/2-ERK-1/2-cyclic AMP-responsive element binding protein pathway, associated with the transition of LNCaP cells to androgen-independence, correlates with the nongenotropic actions of androgen receptor where nuclear translocation and the ability of the androgen receptor to bind to DNA were not prerequisites for androgen receptor activity (21). The challenge therefore will be to identify and understand the actions of such factors and signaling mechanisms not only to better understand the progression of androgen-independent prostate cancer, but also to devise schemes of better therapies for this life-threatening disease.

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## THE ANDROGEN RECEPTOR: UNLOCKING THE SECRETS OF ITS UNIQUE TRANSACTIVATION DOMAIN

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*E. RHOA Effector Protein Kinase*

*C-Related Kinase*

*F. SMRT*

*G. CYCLIN D1*

*H. SHP*

*I. TBL1*

*J. CHIP*

XIV. The Focus on AR NTD

References

Whereas the androgen receptor (AR) protein shares similarities in the structure of its DNA- and hormone-binding domains with other members of the steroid nuclear receptor family, the molecule in its unliganded form has a seemingly unordered amino-terminal transactivation domain unique to the AR. A comprehensive understanding of the specific sub-structures and protein-protein interactions inherent to this domain in both its inactive and activated states remains unachieved. Therefore, the malleability of this peptide region in accommodating the diverse repertoire of transcription-modulating AR cofactors creates a great challenge for those intent on generating relevant three-dimensional molecular models. The AR transactivation domain achieves this flexibility through a series of conformational steps dependent on the presence of cofactors that induce allosteric changes, and thus has evolved several conserved peptide motifs representing key protein-protein interaction surfaces. Elucidation of these signaling regions, including their involvement in inducing AR transactivation domain structural changes, is of foremost interest in understanding how the AR achieves its pivotal role in regulating the androgen signaling axis particularly during the progression of prostate cancer. © 2005 Elsevier Inc.

I. THE ANDROGEN SIGNALING AXIS

Androgens play a key signaling role in a broad range of physiological systems, from influencing the development and maintenance of the immune and neural systems to exerting more obvious control over determining gender-specific phenotypes associated with the male reproductive system and secondary male traits (reviewed in Gelmann, 2002). It is with regard to the physiology of tissues and organs making up the latter that much attention has focused on understanding how androgens mediate cellular growth and differentiation, and how departures from this normally highly regulated signaling axis can lead to abnormal cellular growth, and ultimately cancers of organs such as the prostate. The main androgen in men is testosterone (T), with the testes

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producing about 80–95% of the circulating T and the adrenal glands producing the remaining 5–20%. Carried in the blood bound to sex hormone-binding globulin, T diffuses into the cell and is converted by 5 $\alpha$ -reductase activity into dihydrotestosterone (DHT), the form of the hormone possessing more potent biological activity. Downstream effects of DHT within the cell are then mediated by the androgen receptor (AR) protein, which is present in the cytoplasm but held in an inactive conformation through associations with molecular chaperones such as heat shock proteins. On binding of DHT, the AR undergoes conformational changes that result in dissociation from the chaperones and activation of the receptor. The activated AR translocates into the nucleus, where it coordinates the localized recruitment of transcriptional cofactors to promoters of AR-target genes, thus regulating expression. AR-regulated genes may be positively associated with cellular growth, such as in the case of prostate-specific antigen (PSA), or may be involved in proapoptotic pathways, such as in the case of p21. Cells that are androgen sensitive require the presence of functional AR to properly respond to the hormonal signal. Clinical outcomes resulting from attenuation or loss of AR activity as experienced in patients with complete androgen insensitivity syndrome or in patients undergoing hormone ablation therapy demonstrate the singular importance of the AR in mediating the physiological effects of androgen.

## II. THE ROLE OF AR IN PROSTATE CANCER

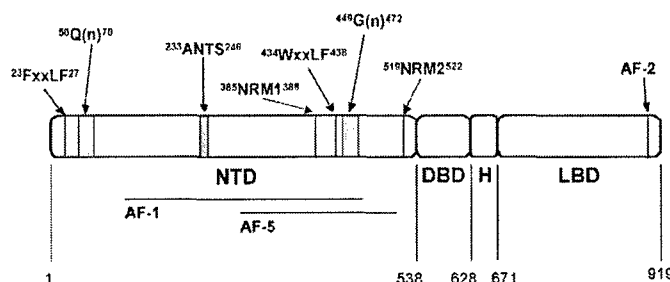
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Prostate cancer is the most common solid tumor found in men from Western countries and is the leading cause of cancer-related death in those populations. Nearly 70% of these cancers involve adenocarcinomas of the peripheral zone, one of four distinct morphological regions of the organ (reviewed in Van de Voorde, 1996). In contrast, enlargement of the prostate resulting from benign prostatic hyperplasia exclusively involves tissues of the transition zone. The development and maintenance of the prostate is androgen dependent, and thus traditional treatment regimens for prostate cancer have involved removal of the testosterone signal. The efficacy of this strategy was first observed in the 1940s, when it was established that men with metastatic prostate cancer experienced tumor regression in response to castration (Huggins and Hodges, 1972). At present, radical prostatectomy or radiation therapy may successfully treat men whose early-detected cancers are clinically confined to the organ capsule. For cases of advanced disease in which tumors have spread to surrounding tissues, hormone ablation therapy involving antiandrogens such as flutamide or bicalutamide have immediate beneficial effects on tumor growth (Taplin and Ho, 2001). However, these ablation strategies are essentially palliative, as within a few years inevitably the tumors return in forms that are more aggressive and unresponsive to the particular hormone treatment (Debes and Tindall, 2004). This condition, known as hormone refractory disease, tragically carries with it a very poor prognosis. Gaining an understanding of

the mechanisms behind disease etiology and subsequent progression from androgen dependence to independence are key focal points of ongoing prostate cancer research. Epidemiologic studies have revealed associations between the size of a polymorphic CAG microsatellite repeat region in the AR gene and the risk of developing prostate cancer, with shorter triplet repeats being associated with increasing risk of disease (Giovannucci *et al.*, 1997; Ingles *et al.*, 1997). This will be discussed in greater depth later in the chapter. Extensive screening studies have cataloged more somatic mutations in the AR of advanced prostate cancer than in any other gene, and it is now estimated that such AR mutations can be identified in up to 40% of advanced-stage cases, with over half of these localized to the AR transactivation domain (Buchanan *et al.*, 2001). It is important to keep in mind, however, that the majority of these somatic AR mutations are believed to emerge only in advanced disease as a result of pressures placed on the tumor by earlier treatments such as ablation therapy. The belief is that exposure to antiandrogens that initially halt the growth of androgen-dependent cells eventually selects for the outgrowth of cell clones containing one or more of the following attributes (reviewed in Balk, 2002; Buchanan *et al.*, 2001; Feldman and Feldman, 2001): somatic AR mutations that allow the receptor to respond promiscuously to other nonnative ligands, become intrinsically active, or interact inappropriately with other signaling molecules; amplified expression levels of AR that create hypersensitivity to lowered levels of androgens; and heightened activation of AR through mechanisms of posttranslational modification of the receptor that normally are tightly controlled—an example would be AR phosphorylation via pathways stimulated by extracellular stimuli such as growth factors or cytokines (Debes and Tindall, 2004). Thus, although prostate cancer development and progression may involve several aberrant cellular pathways, it is abundantly clear that the activity of the AR represents a central hub through which these events pass. A discussion of how the androgen signaling pathways effect broad-ranging changes within the cellular context should begin first with an understanding of how the AR molecule is equipped to take the input signal of androgen and translate that into appropriate output signals that coordinate downstream transcriptional regulation. This chapter takes a closer look at the features of the AR that distinguish it from the other steroid nuclear receptors, with particular interest in discussing the functional roles of signaling motifs found within the transactivation domain of the molecule that are responsible for its activity.

### III. OVERVIEW OF AR STRUCTURE

The AR is a 919-amino acid protein with a molecular weight of about 99 kDa encoded by a single-copy gene located on the X-chromosome at Xq11–12. The AR gene is approximately 80 kb in size and comprises 8 exons



**FIGURE 1.** Functional map of the AR depicting the amino-terminal transactivation domain (NTD), DNA-binding domain (DBD), hinge region (H), and ligand-binding domain (LBD), with amino acid positions at boundaries. Location of the key signaling motifs discussed in the text are indicated.

that collectively possess a coding region of 2757 bp. Similar to other steroid receptors, AR consists of four main functional domains: part of exon 1 codes for the entire amino-terminal transactivation domain (NTD) amino acids ~1–538, exons 2–3 code for a DNA-binding domain (DBD) amino acids ~539–628, and exons 4–8 code for a hinge region amino acids ~629–670, containing a nuclear localization signal (NLS) and the ligand-binding domain (LBD) amino acids ~671–919 (see Fig. 1). For the sake of simplicity, the hinge region is often considered part of the LBD in models that choose to describe the AR as having three main functional regions (i.e., NTD, DBD, and LBD). Along these lines, it should be noted that there is no consensus in the literature as to which specific amino acid residues should be used to conceptually demarcate any of these domain boundaries; this is in part a consequence of the AR possessing polymorphic microsatellite repeats that have resulted in discrepancies in the early sequence publications. As a result, the reader should be aware that it is common in the literature for the AR to be described as consisting of 910 or 917 amino acids in addition to the 919 used in this chapter. Regardless, the numbering system used above is well suited to illustrate the modular nature of the protein and thus provides a convenient strategy for discussing the distinct functional roles carried out by these different domains.

#### IV. THE AR LBD

Substantial tertiary structural information has been obtained for the AR LBD in both its ligand-bound and ligand-unbound states, revealing that despite low homology in amino acid sequence, when compared with certain other steroid receptors, the three-dimensional structures adopted by the LBDs of these molecules show marked similarities (Sack *et al.*, 2001).

Steroid receptor LBDs generally consist of 12 alpha helices, and thus to follow convention, the AR helices are designated 1–12, even though the AR actually lacks the equivalent of what would be LBD helix 2 of the other steroid receptors. Ligands that bind the AR LBD do so by sitting in a ligand-binding pocket defined by helices 4, 5, and 10, which contact the A and C rings of the steroid molecules. On ligand binding, a conformational change takes place in the LBD, whereby helix 12, at the carboxy terminus of the AR, shifts to form a “lid” over the ligand-binding pocket, thus stabilizing the AR-ligand interaction. This has the secondary effect of revealing an otherwise inaccessible protein-protein interaction surface found at the AR carboxy terminal known as activation function 2, or AF-2 (Shiau *et al.*, 1998). This hydrophobic surface serves as the binding site for the p160 family of nuclear receptor coactivators, including TIF2, SRC1, and AIB1, which enhance AR-mediated transactivation through both direct modulations of AR conformation and recruitment of cofactors to the promoter-targeted AR that enhance transcriptional competence (Ma *et al.*, 1999). Another important role of the AR AF-2 motif is that of mediating interactions between the AR NTD and LBD; this AR N/C domain interaction is believed to be a functionally important event in receptor activation following ligand binding (He *et al.*, 1999). A precise understanding of how this AR N/C interaction mechanistically relates to receptor activation has yet to be defined, but ideas concerning its function are discussed in this chapter. For now, it is important to rationalize that the ligand-dependent nature of the AF-2 interaction surface thus imparts ligand dependence on a number of subsequent AR signaling events that occur through this LBD motif.

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## V. THE AR DBD

As with the LBD, there is high functional conservation in the hinge region and DBD of the AR compared to other steroid receptors (Rundlett and Miesfeld, 1995). The main function of the hinge region is that of presenting an NLS that enables targeting of the activated AR to the nuclear membrane. This NLS signal in human AR spans amino acids 618–636 and has the sequence **RKcyeagmtlgARKIKKlg**. The functional consequence of disrupting this NLS has been demonstrated through experiments in which AR with mutations in this region has been shown to localize exclusively to the cytoplasm. Normal translocation of the receptor into the nucleus brings the AR in contact with target chromatin, where the receptor has high affinity for short conserved DNA sequences commonly referred to as hormone response elements found in promoter sites (Luisi *et al.*, 1991). These hormone response elements are specific for respective steroid receptors, and in the case of androgen response elements (AREs), consist of a pair of short hexameric half sites separated by 3-nucleotide spacer elements. The key

identifying feature of the two ARE consensus sequences thus far identified are conserved guanines and cytosines at positions 2 and 5 in each half site of the half-site pair, with only slight differences in the identities of other nucleotides making up the remainder of the sequences. The functional distinction between these two ARE types is not clear, but the relative positioning of these repeats, often in tandem, on AR-targeted DNA indicates that their combined role may not only be to specify the AR-binding site on DNA per se but also to coordinate receptor-receptor synergy of adjacently bound AR (Rastinejad *et al.*, 1995). In fact, the variation in number and relative orientation of AREs present in different AR-regulated gene promoters indicate that subtle modulation of how AR physically binds to the promoter DNA can influence how the receptors mediate transcription. The AR DBD recognizes these ARE sequences via two zinc finger motifs that are separately coded by exons 2 and 3 of the AR gene. These zinc fingers consist of about 25 amino acids each and configure a zinc ion bound to four cysteine residues at the base of each loop, in doing so positioning residues within the DBD for interactions with target DNA. At the base of the first loop there is a short peptide motif amino of acids 577–585, consisting of **G-S-c-K-V-f-f-k-R**, in this case with letters in bold indicating conserved residues among the steroid receptors. This interacts directly with cytosine and guanine at positions 2 and 5 of the target ARE half site. The second loop is involved in enhancing receptor-DNA binding through interacting with the first loop and may play a role in mediating receptor dimerization.

## VI. THE AR NTD

The NTD of the AR makes up over half the amino acid sequence of the complete receptor, a distinction that sets the AR apart structurally from the other related steroid receptors that typically have relatively smaller transactivation domains. As discussed above, the separate functional domains of the AR act in a modular nature. Thus, when the reader regards the schematic representation of the AR domains (see Fig. 1), from right to left the interpretation is that the LBD acts as the on/off switch of the receptor, the hinge region contains the NLS signal responsible for transport of the AR into the nucleus, and the DBD serves as the anchor that positions the AR properly on the promoters of target genes. The role of the NTD, then, is that of subsequently conducting the coordinated recruitment of other proteins that will influence the transcriptional activity that takes place at the promoter, ultimately controlling gene expression. The NTD can be thought of as where the “action” of the receptor occurs, and in a sense the roles of the other AR domains are really to act as regulators of when and where the NTD is allowed to carry out its activity. The importance of the AR NTD is indicated not only by its size in relation to the other AR domains but also by its

complexity and lack of homology with domains of other steroid receptors. Because there is high structural homology of the DBD and LBD of the AR compared to other receptors, the substantial understanding of how these types of domains work in other steroid receptors can be applied to the AR. However, the structure of the AR NTD has remained elusive, primarily because of its being a largely globular domain that may require associations with AR-binding molecules to achieve its mature conformation (Reid *et al.*, 2002). The unique nature of the AR NTD invites much more scrutiny, and thus a detailed discussion of the features that comprise this domain follows.

#### VII. AR AF-1 AND AF-5

In general, The AR NTD transactivation activity is localized to two overlapping activation functions-AF-1 amino acids 142–485, and AF-5 amino acids 351–528 (Jenster *et al.*, 1995)-that encompass a number of peptide features such as microsatellite repeats, protein-protein interaction surfaces, and phosphorylation and sumoylation regulatory sites. The overall structures of these two generalized regions have not been determined, and it is believed that before receptor activation, the entire NTD may exist as a rather large fluid peptide domain in contrast to the highly structured DBD and LBD. On activation of the AR, associations with AR-binding proteins such as coactivators and transcription factors present at the gene promoter may collapse the NTD into structures that are optimal for the transactivation potential of the domain to be realized. Evidence of this mechanism has been shown in the case of the transcription factor TFIIF, which binds AR AF-1 in a manner that induces  $\alpha$ -helical structure formation in the region (Kumar *et al.*, 2004). It is likely that this evolutionary approach of the AR NTD remaining unstructured before protein-protein associations allows for a variety of NTD structures to arise in response to different signaling contexts. One way to think about it is that if the AR is responsible for mediating the wide-ranging responses that cells exhibit to androgens, then clearly the manner in which the AR NTD communicates with other molecules must possess subtlety in its appropriate presentation of output signals. Thus, creation of interaction surfaces in the NTD, where the AR recruits downstream signaling proteins, is allosterically dependent on what upstream AR-binding partners are present to select for their formation. Interestingly, the actions of the two AF regions appear to operate with some independence, as the primary transactivation potential of the NTD in a full-length AR molecule is concentrated at the AF-1 region on ligand stimulation, whereas the focus is shifted to the AF-5 region in a truncated AR that lacks the LBD and is constitutively active. One implication of this finding is that the LBD has an inhibitory role on the NTD in the absence of ligand activation as a means of preventing inappropriate receptor activity, and

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there is evidence that this may involve recruitment of a peptide stretch found just upstream of the DBD in the AF-5 as a means of inhibiting AR-DNA binding (Liu *et al.*, 2003). This is one of several examples of specific peptide features within the AF-1 and AF-5 regions that control the NTD activity, and these will now be discussed in greater detail.

### VIII. Q AND G REPEATS

There are two polymorphic trinucleotide repeats in exon 1 of the AR. The first consists of a stretch of about 21 CAG repeats coding for the amino acid glutamine (Q) spanning amino acids ~58–78, and the second is a stretch of GGN repeats that code for glycines (G) spanning amino acids ~449–472, with the majority of these codons being GGC. In the general population, the size distribution of the AR CAG allele ranges from 6 to 39 repeats but exhibits significant variation among the races, with 65% of African-American males possessing alleles shorter than 22 CAG repeats, compared to 53% of whites and 34% of Asian Americans (Edwards *et al.*, 1999). Our group (Irvine *et al.*, 1995) showed that the presence of shorter CAG and GGC repeats was observed in a high-risk group of African-American men, corroborating previous findings of a genetic component to prostate cancer predisposition. In the laboratory, shorter repeat size has been shown to result in increased activity of the receptor (Chamberlain *et al.*, 1994; Irvine *et al.*, 2000), and likewise complete deletion of the CAG repeat results in a receptor that is much more active than wild-type molecules (Callewaert *et al.*, 2003). It is thus believed that the presence of the Q and G repeats provides inhibitory control over the AR NTD. There is evidence that, just as with similar trinucleotide repeats found in other genes, the AR CAG microsatellites have expanded through the course of evolution (Rubinshtein *et al.*, 1995). Overexpansion in CAG repeat size has detrimental effects on AR signaling. Abnormal repeat sizes of ~40 or more CAG are associated with diseases such as Huntington disease and spinal and bulbar muscular atrophy, which is commonly called Kennedy's disease. This finding has been attributed to the fact that AR molecules with more than 40 Q repeats show markedly decreased transactivational activity in *in vitro* experiments, even though ligand-binding affinities of these molecules are not affected (Chamberlain *et al.*, 1994). In addition, we have recently (Buchanan *et al.*, 2004) provided evidence that the Q repeat size influences the N/C interaction abilities of the AR molecule, with the range of 16–29 Q repeats optimal for AR N/C activity being reflected in 90% of the population having alleles in this range. Parallels exist in the proposed function of the G (encoded by GGC) repeats, with expansions beyond the 16–18 G that represent 90% of the population, resulting in decreased AR function (Ding *et al.*, 2004). Thus, there may have been an evolutionary bias in the expansion of the CAG and

GGC repeats up to an optimal length to favor N/C interaction as a means of AR regulation.

#### IX. AR NTD SIGNATURE SEQUENCE

Throughout evolution, the human AR has maintained over 70% conservation of its DBD and LBD with those from other species. However, this conservation is not present in the NTD, which shares typically less than 50% homology. However, it is notable that a single 14-amino acid NTD sequence spanning amino acids 233–246 is absolutely conserved in the AR across all species for which coding sequence is known. This sequence, AKELCK-AVSVMGL, is not present in any of the other steroid receptors, and thus most likely, it is the product of convergent evolution among species. Considering the suggested functional importance of this sequence by its unusually faithful conservation, and the fact that the AR NTD signature sequence (ANTS) sequence lies within the AF-1 region, an important role of ANTS in regulating AR transactivation activity seems highly likely. There is evidence that ANTS serves as an interaction site for heat shock proteins that act to stabilize the AR in its inactive state, and thus may represent a built-in inhibitory region that represses or deactivates receptor activity (He *et al.*, 2004). Further understanding of the functional nature of this motif and identification of additional AR cofactors that use it for receptor interaction are needed.

#### X. FxxLF AND WxxLF MOTIFS

As mentioned previously, the p160 coactivators are recruited to the AR via the DHT-induced AF-2 interaction surface of the AR LBD. The p160s possess a series of short LxxLL peptide motifs (where L is leucine and x is any other amino acid), commonly referred to as nuclear receptor boxes (NR-boxes) that directly contact the hydrophobic cleft of the AR AF-2. The Wilson group (He *et al.*, 2000) identified two LxxLL-like motifs present in the AR NTD that are highly conserved across the AR of many species, and they demonstrated that these motifs mediate interactions of the NTD with the LBD via the same AF-2 binding site used by the p160s. The first is a <sup>23</sup>FxxLF<sup>27</sup> motif (in humans FQNLF) that has greater affinity with the AF-2 than that of the p160s. Binding of the FxxLF with the AF-2 results in the aforementioned N/C interaction that may displace (out-compete) p160 binding to the AF-2. However, the p160 molecule may still remain associated with AR through alternate binding sites in the AR NTD, localized roughly to the C-terminal third of the NTD amino acids ~351–538 (Irvine *et al.*, 2000; Ma *et al.*, 1999). The role of N/C interaction is not clear, but the

prevailing hypothesis is that this conformational change in response to DHT binding facilitates the activation of the receptor by revealing the protein-protein or protein-DNA interaction surfaces that subsequently result in dimerization, DNA binding, and receptor transactivation. Similarly, the <sup>434</sup>WxxLF<sup>438</sup> motif (in humans WHTLF) is also involved in mediating N/C interaction, albeit much less efficiently than FxxLF. This WxxLF-mediated interaction does not involve the LBD AF-2 but, rather, some other region of the LBD that is yet to be determined. However, the primary role of the WxxLF may be to recruit the p160s to the AF-5 region of the NTD, as evidenced by decreased p160-AR binding to the NTD following mutation of the WxxLF motif.

#### XI. PHOSPHORYLATION OF THE AR NTD

The newly synthesized AR quickly undergoes posttranslational modification via phosphorylation, with the resulting modified form of the receptor adopting a higher molecular weight of about 110 kDa. Although it has been observed that the AR is phosphorylated at about a dozen serine residues throughout the receptor, the majority of these sites are located within the NTD and include constitutively phosphorylated positions such as serine 94, as well as those that undergo phosphorylation associated with receptor activation such as at serine positions 16, 81, 256, 308, and 424, as described by the Weber group (Gioeli *et al.*, 2002). There is quite a bit of evidence to indicate that phosphorylation is a normal means of regulating AR signaling both directly and indirectly. Of particular interest are pathways that can potentially activate the receptor without the binding of ligand that may be amplified in advanced hormone refractory tumors. These pathways involve extracellular stimuli such as growth factors, cytokines, and HER-2/neu acting through multitiered kinase signaling cascades that result in AR phosphorylation (Wen *et al.*, 2000). Thus, these different, yet often overlapping, pathways have been extensively investigated for their regulatory roles on AR activity.

Activators of protein kinase A have been shown to stimulate expression of the AR-mediated PSA gene independent of androgen in prostate cancer derived cell lines. The dose-dependent increase in PSA observed in these experiments was conditional on the presence of AR, as evidenced by inhibition of expression when the antiandrogen bicalutamide was introduced into the cells to interfere with AR function (Nazareth and Weigel, 1996). It would be inaccurate, though, to state that the functional consequences of phosphorylation on AR activity are necessarily stimulatory. As an example, the cytokine interleukin 6 has been shown to be both an AR activator as well as a repressor *in vivo* (Jia *et al.*, 2003, 2004). In addition, serine 515, which is near the carboxyl end of the AF-5, has been shown to be a phosphorylation

target of the MAPK signal cascade. Interestingly, experiments conducted using forskolin, a known stimulant of the MAPK pathway, have yielded conflicting results; LNCaP prostate cancer cells, when treated with forskolin, have been shown to exhibit enhanced AR activity, as indicated by increased expression of endogenous PSA (Jia *et al.*, 2003), whereas this same cell line has been used to demonstrate that forskolin induces dephosphorylation of the AR, resulting in impaired ligand binding affinity (Blok *et al.*, 1998). Seeming discrepancies in interpreting outcomes of AR phosphorylation in the laboratory are certainly in part a result of the lack of understanding of how the different AR serine positions available for phosphorylation are individually relevant. It is also quite likely that the overlapping nature of kinase cascades within the cell make it difficult for researchers interested in characterizing these pathways to isolate specific effects. Further work is needed to elucidate the complex nature of these mechanisms that act on the AR, but in the meantime it is clear that research focused on these nonsteroidal modes of AR activation may provide great insight into the progression of prostate cancer from androgen dependence to hormone refractory disease.

## XII. SUMOYLATION AT NRM1 AND NRM2

The AR is posttranslationally modified by the addition of the small, ~100-amino acid protein SUMO-1 at two lysine residues in the receptor in a process referred to as sumoylation (Poukka *et al.*, 2000). These two NTD sites were originally identified as negative regulatory motifs, and thus are referred to here as NRM1 (<sup>385</sup>IKLE<sup>388</sup>) and NRM2 (<sup>519</sup>VKSE<sup>522</sup>), both of which exhibit sequences closely matching the consensus for targeting by the Ubc-type enzymes that serve as SUMO-E3 ligases (Iniguez-Lluhi and Pearce, 2000). This process is highly reminiscent of ubiquitination, in that homologous E1, E2, and E3 enzymes facilitate the activation and transfer of SUMO-1 to appropriate target substrates. Unlike ubiquitination, however, which results primarily in the irreversible flagging of proteins for degradation via the 26S proteasome machinery, sumoylation is a reversible process that mediates several different effects on targeted proteins. Such outcomes include activation, repression, intracellular localization, and degradation.

In the case of AR, proteins belonging to the family of protein inhibitors of activated STAT (PIAS) serve as SUMO-E3 ligases. Two members of this group, PIAS1 and PIASxalpha, have been shown to repress ligand-dependent transactivation of AR concordant with enhancement of receptor sumoylation (Nishida and Yasuda, 2002). Interestingly, the activation of the AR by ligand binding appears to be a prerequisite for sumoylation, a finding that implicates the covalent attachment of SUMO as a means of deactivating

AR molecules that are already engaged in transcriptional signaling. Deletion of the NRM1/2 sites in the NTD relieve the AR from SUMO-mediated repression, as does the activity of the AR-interacting protein Zimp10, which collocates with SUMO-bound AR and acts as an AR coactivator (Sharma *et al.*, 2003). Although the current understanding of AR sumoylation still leaves much to be desired, it is clear that this mechanism represents another layer of regulatory control targeted at the NTD; the positioning of these NRM motifs in the AF-5 subdomain raises additional questions as to the signaling properties of this receptor region.

### XIII. AR NTD-ASSOCIATED COFACTORS

If it is true that the main function of the AR-NTD is to provide a staging platform for cofactor recruitment at the promoters and enhancers of target genes, the main questions are, What are these cofactors, and how do they function? A number of cofactor candidates can be considered (a subset of all reported ones) based on the fact that they are known to interact with the AR NTD and that they make physiological sense in terms of AR activity modulation.

#### A. p160 COACTIVATORS

Three related proteins with multiple names (SRC-1, GRIP1/TIF2, and pCIP/ACTR/RAC3/AIB1/TRAM1) belong to this family and collectively are the best characterized of the nuclear receptor coactivators (reviewed in Rosenfeld and Glass, 2001). Each one has multiple LxxLL motifs, which bind to the AF-2 region of all nuclear receptors. In the AR, an additional strong interaction surface for binding of p160 coactivators exists in the AF-5 region of the AR (Irvine *et al.*, 2000). It is therefore possible that p160 cofactors bridge AR N/C interactions.

#### B. BRCA1

Mutations in BRCA1 have been associated with familial breast cancer susceptibility. Although functions for BRCA1 in DNA repair have been proposed, it was shown that BRCA1 represses the activity of the ER (Fan *et al.*, 1999). However, we have demonstrated that BRCA1 functions as a coactivator for AR (Park *et al.*, 2000). In addition, we have shown that BRCA1 interacts with the AR primarily through the AF-5 region of the NTD (unpublished observations). Although the mechanism is unknown, this function of BRCA1 indicates that the AR may mediate the tumor-suppressor or growth-regulatory effects of BRCA1.

### C. p300/CBP

These factors are secondary coactivators, normally recruited to the preinitiation transcription complex by primary coactivators such as the p160 family members. They provide necessary histone acetylation activity for the conversion of chromatin to transcriptionally permissive local structures. In addition, binding sites for CBP/p300 exist in AF-5 of the AR. It is unclear whether p300 and CBP play redundant or complementary roles with respect to each other.

### D. ARAS

Chang and colleagues (Rahman *et al.*, 2004; Sampson *et al.*, 2001) have identified AR-associated proteins that interact with the AR NTD (ARA24, ARA160/TMF). ARA24 or RAN interacts with the Q repeat and also might be involved in AR trafficking in/out of the nucleus.

### E. RHOA EFFECTOR PROTEIN KINASE C-RELATED KINASE

Recently, protein kinase C-related kinase was identified as a transcriptional activator of the AR via nonsteroidal superactivation and physical interaction with the AF-5 subdomain (Metzger *et al.*, 2003).

### F. SMRT

This corepressor, originally discovered by the Evans laboratory, acts to inhibit nuclear receptor-mediated transactivation activity (reviewed in Rosenfeld and Glass, 2001). It interacts with both the AR LBD and AR NTD (Dotzlaw *et al.*, 2002).

### G. CYCLIN D1

This protein is a required component of the CDK4 complex that plays a role in cell cycle control via phosphorylation of the retinoblastoma tumor suppressor gene (Harbour and Dean, 2001). Cyclin D1 binds directly to the AR NTD and inhibits AR transactivation activity (Petre *et al.*, 2002). It is not known exactly which regions or subdomains in the AR NTD are necessary for cyclin D1 binding or how structural alterations in the AR might affect the binding of cyclin D1 and its AR inhibitory activity.

### H. SHP

The SHP—short heterodimer partner—protein is an orphan receptor that lacks a DNA binding domain and was recently shown to interact with the AR NTD, causing an inhibition of AR-mediated transactivation activity

(Gobinet *et al.*, 2001). Apparently the inhibition is caused by competition with AR coactivators like GRIP1/TIF2.

### I. TBL1

TBL1, an F box/WD-40-containing factor forms a crucial part of the corepressor/coactivator exchange complex required for transactivation activity of many steroid receptors, including the AR (Perissi *et al.*, 2004). It may serve as an adaptor for the recruitment of the ubiquitin-conjugating/19S proteasome complex and thus may play a vital role in modeling factors on/off from transcription complexes.

### J. CHIP

This COOH terminus of the Hsp70-interacting protein was identified by the Wilson laboratory (He *et al.*, 2004) as interacting with the ANTS sequence in the AR NTD (see earlier). CHIP functions as a negative regulator of AR transcriptional activity by promoting AR degradation.

Collectively, these observations indicate that optimal AR transactivation requires interaction of AR with an appropriate assembly of AR associated cofactors, and that if they are expressed in an inappropriate manner, AR-mediated gene expression can be driven by nonsteroidal mechanisms in the absence or at extremely low levels of androgens. The focus of future research is to define their binding sites in terms of the sequence motifs referred to earlier and how they effect AR interdomain interactions and activity.

## XIV. THE FOCUS ON AR NTD

The AR NTD is unique among steroid receptors both in its expanded size and lack of determined form before activation. Although the latter fact has made gaining insight into the various NTD signaling features challenging on the basis structural homology comparisons, it also indicates that the ability of the AR to coordinate wide-ranging cellular responses to androgens may be a direct result of highly evolved structural flexibility. Unraveling the functional mechanisms of the NTD, from the nature of the N/C interaction to the roles of receptor phosphorylation and sumoylation, is a goal of great importance if we are to better understand how the AR is able to act as the central unifying molecule in the varied cellular pathways of androgenic response. Likewise, we now understand that the AR may still be playing the primary signal-mediating role in cases of hormone refractory prostate cancer resulting from amplification of secondary, nonligand dependent pathways. This is currently a focal point of research concerned with explaining how certain tumors that start as androgen-dependent tumors

can progress beyond the need for hormone stimulus with tragic results. The sheer number of new incidence cases of prostate cancer every year translates into a physical, financial, and certainly emotional burden for men and their loved ones that is perhaps unrivaled in male medicine. It is of paramount importance that researchers continue to learn about not only the etiology but also the nature of prostate cancer progression that so often turns from seemingly successful initial treatment to the rapid return of tumors that no longer respond to androgen ablation strategies. The AR is central to this research, and as we continue to gain better understanding of its molecular functions, undoubtedly we will see this knowledge translated into greater medical benefits.

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Mini review

# Molecular chaperones throughout the life cycle of the androgen receptor<sup>☆</sup>

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## Abstract

Aberrant signaling by the androgen receptor contributes to the initiation and progression of prostate cancer. The involvement of molecular chaperones in the processes of folding, activation, trafficking, and transcriptional activity of the androgen receptor provide different points along the signaling axis where regulation of androgen receptor activity can be hijacked to provide growth signals for clonal selection in cancer progression. Evidence exists of abnormal chaperone expression that could contribute to the upregulation of AR activity in prostate tumors. Regardless of whether chaperones are involved in the causation of prostate carcinogenesis, molecular chaperones provide therapeutic targets for the treatment of prostate cancer.

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## 1. Introduction

The androgen receptor (AR) is a transcription factor belonging to the class I subgroup of the nuclear receptor superfamily. Members of this superfamily are ligand-responsive and share structural and functional similarities with one another [1]. Stimulated by androgens, the AR signaling pathway plays an

important role in the development and differentiation of target tissues [1,2], as well as a critical role in the initiation, proliferation, and progression of prostate cancer to ligand-independent disease [3,4].

The predominantly cytoplasmic AR rapidly translocates into the nucleus in response to androgen [5,6]. As with any signal transduction pathway, proper signaling is dependent upon the receptor's ability to acquire more than one conformational state [7]. Unbound to ligand, the AR is maintained in an inactive, but highly responsive state by a large dynamic heterocomplex composed of heat shock proteins, co-chaperones, and tetratricopeptide repeat (TPR)-containing proteins [8]. Most of the androgen receptor-chaperone interactions identified so far appear to involve the ligand-binding domain (LBD)

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of the receptor. Deletion of the AR-LBD abolishes hormone-responsiveness, and results in the constitutive activity of the receptor [2,5]. Therefore, neither the LBD nor hormone is believed to be necessary for AR transactivation activity. Instead, the primary function of the LBD may be to inhibit activation of the AR and, therefore, the transcriptional activation of its target genes in the absence of hormone. Binding of 5 $\alpha$ -dihydrotestosterone (DHT) to AR relieves the inhibition imposed by the LBD [2].

The molecular chaperone complex is believed to configure the ligand-binding domain into a relatively stable, partially unfolded, inactive intermediate with a high-affinity for DHT [7,8]. Hormone binding to the complex permits the folding of the AR into an active conformation [2,8]. Binding of ligand, followed by the dissociation of the receptor-chaperone complex and activation of the receptor is viewed as the general regulatory mechanism of AR signaling [9]. However, molecular chaperones remain important players in the events downstream of receptor activation, and throughout the life cycle of the AR (see below).

## 2. Minimal molecular chaperone complex (Fig. 1)

Initially identified by their accumulation in response to cellular stress, chaperone proteins are able to recognize and bind to hydrophobic regions on unfolded or partially folded proteins preventing their irreversible aggregation, promoting cycles of chaperone-mediated folding instead [10,11]. However, even under normal cellular conditions molecular chaperones assist in the proper folding of steroid hormone receptors. The structural conformation of the LBD required for AR activation is accomplished through multiple cycles of binding and release of the AR by the components of the multichaperone complex [7,10,12]. Without these chaperone proteins, the receptor is not denatured. Instead the receptor acquires a conformation where the hydrophobic pocket within the ligand-binding domain remains inaccessible to hormone, preventing activation. Therefore, the receptor is required to be in a partially unfolded high-affinity ligand-binding conformation stabilized by the chaperone heterocomplex in order to be responsive to stimuli [13].

Much of the work on the chaperone-steroid hormone relationship prior to the 1990s did not

specifically focus on the AR. Prior studies had identified receptor-Hsp90 heterocomplexes for the glucocorticoid receptor (GR), progesterone receptor (PR), mineralocorticoid receptor (MR), and estrogen receptor (ER), which sediment at  $\sim$ 9S [8]. The highly conserved LBD among nuclear receptors [14] and the existence of a  $\sim$ 9S form of AR [8] suggested that a similar AR-Hsp90 heterocomplex existed as well. Later studies confirmed that Hsp90 does in fact interact with the LBD of the AR along with Hsp70 and Hsp56/FKBP52 [15,16], which were required to maintain the AR in a high affinity ligand-binding state that is hormone- [17] and temperature-dependent [18,19].

The basic heterocomplex, dubbed the 'foldosome', generally required for the efficient folding and stabilization of steroid hormone receptors consists of Hsp70 (hsc70), Hsp40 (Ydj1), Hop (p60), Hsp90, and p23 [20, 21]. This minimal complex is believed to be essential for ligand responsive signaling of all full-length steroid hormone receptors [7]. Subcomplexes of chaperone proteins lacking receptor or other substrate peptides have been found to exist in the cytosol [22]. However, the assembly of steroid hormone receptors into the ligand competent state is believed to occur in an assembly-line type of process [8,23,24]. Having been shown to associate with ribosomes during translation [25], the first chaperone protein likely to interact cotranslationally with the AR is Hsp70 along with its co-chaperone Ydj1, a DnaJ-like member of the Hsp40 family. Ydj1 augments Hsp70's ability to reversibly bind to small stretches of hydrophobic amino acids [10,12,26] on the nascent peptide to maintain the receptor in a soluble state, preventing irreversible aggregation [27]. Ydj1 may actually bind to the non-native peptide prior to Hsp70 binding to facilitate the transfer of AR to Hsp70 [28]. Ydj1 is hypothesized to potentially assist in correctly aligning the Hsp70 and receptor interaction surfaces [11].

After the initial binding of Hsp70 and Ydj1 to the receptor, Hip/p48 (hsc70 interacting protein), another Hsp70 co-chaperone, binds to stabilize and prolong the interaction between the receptor and this intermediate chaperone complex [29]. Hop (Hsp organizer protein) then binds to the complex and recruits a homodimer of Hsp90 [30] by physically forming a bridge between Hsp70 and Hsp90 using its TPR motifs in the interactions. In addition, Hop contributes

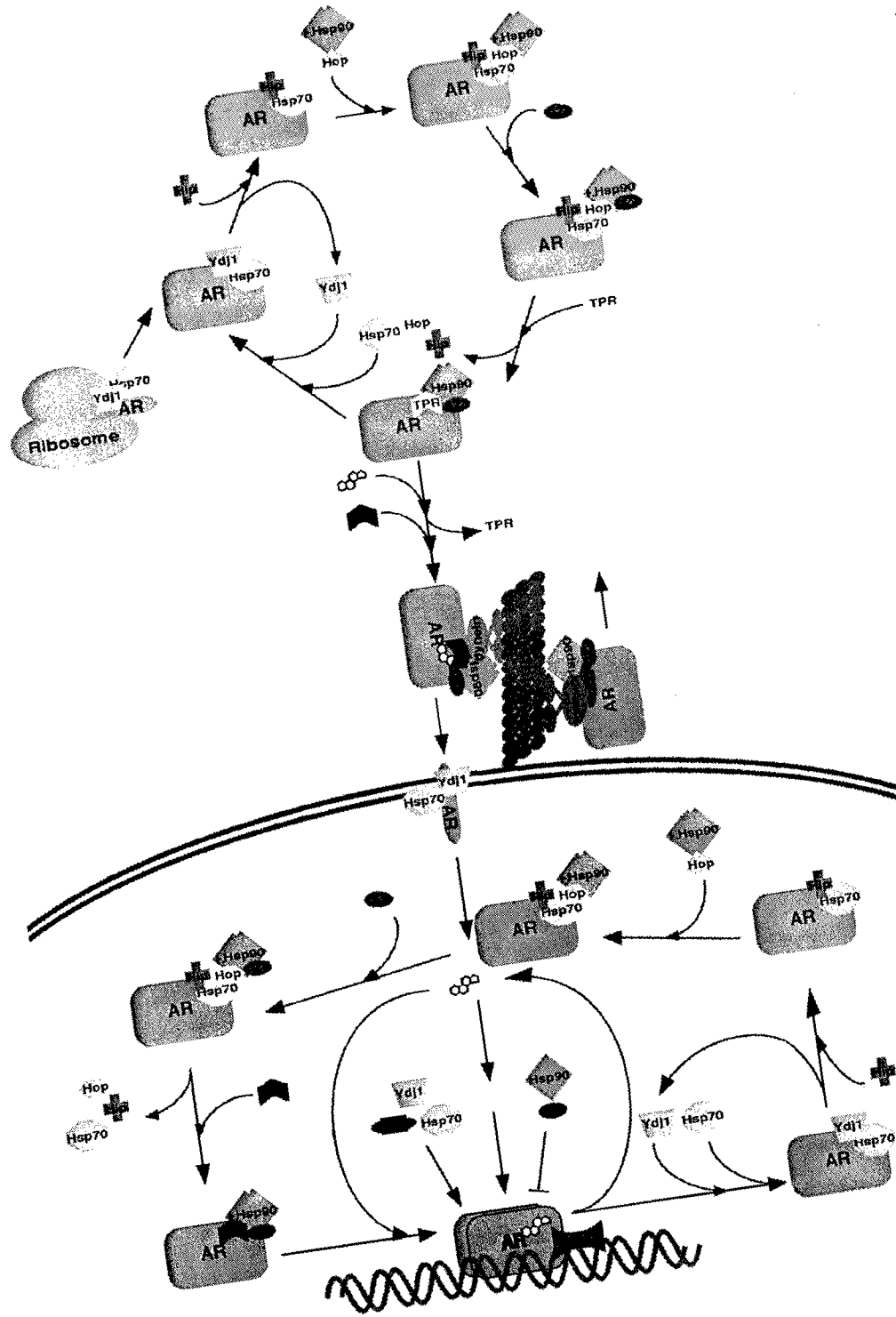


Fig. 1.

to more efficient folding by influencing conformational changes within Hsp70 and Hsp90 [13]. To stabilize the AR in the high-affinity, inactive intermediate conformation, p23, a small molecular weight chaperone, binds to Hsp90 within the complex as Hip, Hop, and Hsp70 dissociate [30–33]. The release of Hop allows for the interaction of a TPR-containing immunophilin with Hsp90, generating the ‘final complex’ [9].

The interactions between steroid hormone receptors and molecular chaperones are transient [19,34] resulting in a dynamic folding/refolding cycle [13] driven by conformational changes within Hsp90 [32]. In the absence of hormone, an equilibrium is established which maintains the AR in an overall high-affinity ligand-binding state. Hormone binding causes the receptor to undergo a sequential loss of chaperones, a process thought to occur as a result of blocking the formation of the receptor-Hsp70 complex and, therefore, the subsequent interactions that occur in the assembly/disassembly cycle of the disactivation loop [19]. With the assistance of Hsp90 [32], transformation of the receptor into the DNA-binding competent state [16] is followed by nuclear translocation, recruitment of cofactors, and transcription of target genes [32].

### 3. AR Translocation (Fig. 1)

Based on studies of PR, ER, and GR, it is known that steroid hormone receptors undergo continuous nucleocytoplasmic shuttling [35] with the predominant localization determined by the accessibility of the nuclear localization signal (NLS) [36]. The NLS of the AR, assumed to be hidden in the inactive conformation, is unmasked upon receptor activation allowing transport into the nucleus [37]. Nuclear transfer of the AR has been shown to be hormone concentration- [6] and Hsp90-dependent [32].

A recent study proposes that the earliest step in receptor activation is not the dissociation of the heterochaperone complex, but the exchange of one TPR-containing immunophilin for another along with the recruitment of cytoplasmic dynein [31]. This, in conjunction with the reported finding of an Hsp90 interaction with the cytoskeleton [12], provides a mechanism for the translocation and accumulation of

the AR within nuclear foci [37,38]. The model developed proposes the involvement of Hsp90 complexes in the trafficking of the receptor along microtubules, using the particular TPR-containing protein associated with the heterocomplex as a guide to sites of receptor action [39]. Once the receptor is activated, the association of an immunophilin with the Hsp90 complex is thought to guide the receptor in a retrograde direction along microtubules. Whereas, binding of Cdc37 (p50) is believed to be the Hsp90-associated protein that is responsible for anterograde trafficking [13,36].

Hsp70 proteins have also been found to associate with steroid hormone receptors in the presence of ligand [40] and are thought to play a role in translocating the receptor across intranuclear membranes presumably by temporarily unfolding the receptor [12,41]. Ydj1, a putative component of the matrix lamina pore complex, may assist Hsp70 in unfolding/refolding the AR as the receptor is transported across the nuclear membrane [42]. Once inside the nucleus, the chaperone complex dissociates from the receptor [31].

### 4. Transcriptional activation

In addition to the influence of cofactors on AR activity, there is also evidence for molecular chaperone involvement in the activation of target genes. Bag-1 proteins, which are Hsp70 co-chaperones, are involved in hormone receptor transactivation. Specifically Bag-1L, the long, constitutively nuclear isoform of the Bag-1 proteins [43] has been shown to complex with the AR in the presence of hormone to upregulate its activity [44]. Bag-1L may act directly on the transcription factor complex as it was found to associate with androgen response elements in the absence of hormone. With recruitment of AR and Hsp70 to the PSA promoter, Bag-1L could perhaps assist these proteins in conformational changes that enhance interactions between the NH<sub>2</sub>-terminal and the COOH-terminal regions of the AR [24]. Bag-1L could also contribute to an increase in transcriptional activity through the recruitment of coactivators to the transcription regulatory complex [24], as well as potentially facilitate the cross-talk of AR with alternate signaling pathways via its association with

kinases [43]. However, this stimulatory effect of Bag-1L on AR activity cannot be generalized to all steroid hormone receptors. In contrast to AR, studies have shown that Bag-1L inhibits GR activity [45].

Cdc37, which was initially thought to be exclusively associated with kinase-chaperone complexes because of the lack of an association with GR [46], was later shown to directly interact with the LBD of the AR [40]. Cdc37 appears to influence AR transactivational activity in a partially hormone-dependent manner downstream of hormone binding [40,47], possibly by assisting the receptor in acquiring conformational changes required for activation. This effect cannot be inferred to all steroid hormone receptors as the loss of Cdc37 function did not have much of an effect on GR activation [47].

Additional chaperone proteins that have exhibited a role in AR transactivation activity, independent of their roles in previous folding processes, include Ydj1 as a stimulatory factor [48], and p23 as an inhibitory factor on AR activity. The inhibitory effect of p23 is yet another example of receptor-specific regulation since p23 was found to increase the transcriptional activity of GR [30]. Why these co-chaperone proteins exert differential effects with respect to the associated receptor is unknown. Even though steroid hormone receptors interact with the same minimal chaperone complex, it may be the absolute composition of the receptor-chaperone heterocomplex, with all of its associated proteins, that dictates the functional activity of the receptor.

## 5. Transcription complex disassembly (Fig. 1)

As mentioned above, the heterocomplex dissociates from the receptor once it has translocated into the nucleus. Yet, molecular chaperones remain implicated in receptor activity for the disactivation loop is believed to occur within the nucleus as well. A recent model proposed that molecular chaperones are involved in the disassembly of transcription factor complexes in order to promote the dissociation of hormone from the receptor and reassemble the receptor in the inactive state. Thus, if DHT is still present, reactivation of the AR occurs. However, if the hormone is no longer around, the receptor reenters the disactivation loop. In this manner, intranuclear

cycling provides a transcriptional regulatory mechanism that is capable of detecting declining levels of ligand, resulting in the rapid termination of receptor activity once the signal is abolished [13,49]. In testing this disassembly model, it was revealed that p23, and possibly to a lesser extent Hsp90, actively disassembled regulatory complexes from DNA elements [50].

## 6. Degradation

Steroid hormone receptor degradation occurs via the ubiquitin-proteasome pathway [13]. When a receptor is unable to successfully transition into the high-affinity ligand-binding conformation the receptor undergoes degradation. CHIP (carboxyl terminus of Hsc70-interacting protein), an Hsp70 co-chaperone protein, has been shown to directly interact with the AR [51] and Hsp90 in the apo-receptor complex, causing the dissociation of p23 and loss of steroid binding ability [52]. Ubiquitylation is induced, followed by degradation via the proteasome [51–53]. CHIP-mediated degradation of the AR [51,53] has been put forth as a quality control mechanism to prevent protein aggregation [54]. CHIP may also prevent the accumulation of structurally unsound AR through the reduction in the rate of AR synthesis [53].

## 7. Chaperones in prostate cancer

An increase in certain chaperone proteins has been documented for prostate cancer. The basal cells of a benign prostate normally express Bag-1L, but the epithelial cells do not. However, in prostate cancer, the opposite is observed where Bag-1L is expressed in malignant epithelial cells and is no longer expressed in the basal cells. The distribution of Bag-1L is similar to Hsp70, which could possibly act synergistically to increase AR transcriptional activity [24]. Cdc37 is another chaperone protein that has displayed increased expression in carcinomas. Animal studies suggest a potential early role for Cdc37 in prostate cancer development. Overexpression of this protein in animal models has been associated with abnormalities such as prostatic epithelial cell hyperplasia and dysplasia [55]. In the human male, abnormal



expression of these proteins may act to enhance androgen receptor action providing a selective advantage to tumor cells during androgen ablation therapy [44].

## 8. Clinical implications

Alterations in the receptor-chaperone complex may well lead to aberrant AR signaling that could contribute to prostate carcinogenesis [24]. As described above, overexpression of certain molecular chaperones would enhance androgen receptor activity. Whereas a downregulation of inhibitory chaperones such as CHIP, which has a putative role in maintaining low levels of AR in the normal prostate [51], would contribute to progression via an accumulation of the receptor, resulting in an upregulation of AR action.

However, even in the absence of a causal role for chaperones in prostate cancer development, molecular chaperones serve as potential therapeutic targets in prostate cancer treatment. Currently, Hsp90 is a therapeutic target in prostate cancer clinical drugs trials [56]. Inhibition of Hsp90 with drugs such as geldanamycin prevents the stable association of p23 with the receptor-chaperone complex, therefore, trapping the AR in an intermediate state [57]. The persistent interaction between Hsp90 and steroid hormone receptors shifts the balance between folding and degradation toward degradation [13]. Targeting chaperone proteins in combination with AR antagonists or radiation may have a synergistic effect in impeding AR activity in prostate cancer [32,58].

## 9. Conclusion

Molecular chaperones play key roles in the folding, translocation, activation, and degradation of steroid hormone receptors depending on whether the receptor is in its inactive or active state [33,48]. There is a core minimal complex involved in the folding of steroid hormone receptors. When activated, the conformational state of the AR may recruit specific TPR-containing proteins depending on its trafficking and/or functional needs [10,30,34]. Competition for the AR

between stimulatory factors and inhibitory molecular chaperones such as p23 [50] could influence the rate of disassembly of the transcription factor complex and, in turn, have an impact on the receptor's ability to rebound to the promoter and initiate another round of transcription.

Altered expression and/or function of molecular chaperones have the potential of augmenting AR signaling, which would then contribute to prostate carcinogenesis. One of the main molecular chaperones, Hsp90, is already being pursued as a potential therapeutic target. However, due to the ubiquitous nature of this chaperone, the potential for side effects as a result of targeting this protein is great. To minimize potential toxicities, efforts may need to be focused on discovering and targeting co-chaperone and chaperone-associated proteins, which have specific functional effects on the AR, but do not disrupt the signaling pathways of the other steroid hormone receptors.

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